FORM PTO-1390 (REV. 11-2000)	U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY'S DOCKET NUMBER
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		3298.1000-000
		U.S. APPLICATION NO. (If known, see 37 CFR 1.5) **09/936507**

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/CA00/00246	09 March 2000	15 March 1999

TITLE OF INVENTION

ELISA KIT FOR THE DETERMINATION OF METABOLIC PHENOTYPES

APPLICANT(S) FOR DO/EO/US

Brian Leyland-Jones et al.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.

2. [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.

3. [ ] This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.

4. [X] The US has been elected by the expiration of 19 months from the priority date (Article 31).

5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
    a. [ ] is attached hereto (required only if not communicated by the International Bureau).
    b. [X] has been communicated by the International Bureau.
    c. [ ] is not required, as the application was filed in the United States Receiving Office (RO/US).

6. [ ] An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
    a. [ ] is attached hereto.
    b. [ ] has been previously submitted under 35 U.S.C. 154(d)(4).

7. [X] Amendments to the claims of the International Aplication under PCT Article 19 (35 U.S.C. 371(c)(3)).
    a. [ ] are attached hereto (required only if not communicated by the International Bureau).
    b. [ ] have been communicated by the International Bureau.
    c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
    d. [X] have not been made and will not be made.

8. [ ] An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).

9. [ ] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).

10. [ ] An English lanuage translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 20 below concern document(s) or information included:

11. [ ] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.

12. [ ] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

13. [ ] A FIRST preliminary amendment.

14. [ ] A SECOND or SUBSEQUENT preliminary amendment.

15. [ ] A substitute specification.

16. [ ] A change of power of attorney and/or address letter.

17. [ ] A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.

18. [ ] A second copy of the published international application under 35 U.S.C. 154(d)(4).

19. [ ] A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).

20. [ ] Other items or information:

U.S. APPLICATION NUMBER 09/936507	INTERNATIONAL APPLICATION NO PCT/CA00/00246	ATTORNEY'S DOCKET NUMBER 3298.1000-000

21. ☒ The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):

	CALCULATIONS PTO USE ONLY

Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO $1000.00

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO $860.00

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO $710.00

International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) $690.00

International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) $100.00

ENTER APPROPRIATE BASIC FEE AMOUNT = | $ 860.00

Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)). | $

CLAIMS	NUMBER FILED	. NUMBER EXTRA	RATE	$
Total claims	95 - 20 =	75	x $18.00	$ 1350.00
Independent claims	30 - 3 =	27	x $80.00	$ 2160.00
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ $270.00	$
	TOTAL OF ABOVE CALCULATIONS =			$ 4370.00

☐ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2. + | $

SUBTOTAL = | $ 4370.00

Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)). | $

TOTAL NATIONAL FEE = | $ 4370.00

Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). $40.00 per property + | $

TOTAL FEES ENCLOSED = | $ 4370.00

Amount to be refunded:	$
charged:	$

a. ☒ A check in the amount of $ __4370.00__ to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of $ _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. __08-0380__. A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:
David E. Brook, Esq.
Hamilton, Brook, Smith & Reynolds, P.C.
Two Militia Drive
Lexington, Massachusetts 02142
United States of America

David E. Brook
SIGNATURE

David E. Brook
NAME

22,592
REGISTRATION NUMBER

IN THE UNITED STATES RECEIVING OFFICE (RO/US)

Designated/Elected Office (DO/EO/US)

U.S. National Phase of
.International Application No.: PCT/CA00/00246

International Filing Date: 09 March 2000

Priority Date Claimed: 60/124,488, filed 15 March 1999

Applicants: Brian Leyland-Jones and Pierre Wong

Title: Elisa Kit For The Determination Of
                                 Metabolic Phenotypes

Attorney's Docket No.:   3298.1000-000

DATE: _12 September 200 1_

EXPRESS MAIL NO. _EL 762236098US_

ELISA KIT FOR THE DETERMINATION OF METABOLIC PHENOTYPES

BACKGROUND OF THE INVENTION

(a) Field of the Invention

5   The invention relates to an enzyme linked immunosorbent assay (ELISA) kit for the rapid determination of metabolic phenotypes including but not limited to the following enzymes, CYP 1A2, N-acetyltransferase-1 (NAT-1), CYP 2D6, CYP 2E1, and 10   CYP 3A4. The ELISA kit uses may include but not be limited to, use on a routine basis in a clinical laboratory, and allowing a physician to a) individualize therapy for the numerous drugs metabolized by these enzymes, b) to predict 15   susceptibility to carcinogen induced diseases including many cancers, and c) to reduce the number of patients undergoing clinical testing by selecting for patients with the appropriate phenotype most likely to respond.

(b) Description of the Prior Art

20   For the majority of drugs (or xenobiotics) administered to humans, their fate is to be metabolized in the liver, into a form less toxic and lipophilic with their subsequent excretion in the urine. Their metabolism involves two systems which act 25   consecutively: the cytochrome P450 system which includes at least 20 enzymes catalyzing oxidation reactions and localized in the microsomal fraction, and the conjugation system which involves at least 5 enzymes. An enzyme of one system can act on several 30   drugs and drug metabolites. The rate of metabolism of a drug differs between individuals and between ethnic groups, owing to the existence of enzymatic

polymorphism within each system. Two or three phenotypes can be distinguished: poor metabolizers (PM), extensive metabolizers (EM), and ultra-extensive metabolizers (UEM). Knowledge of the phenotype is useful clinically because:

a)   the phenotype is associated with toxicities in chemical plants, diseases and cancers.

b)   it allows physicians to prescribe a drug regimen on the individual basis.

c)   it provides a rationale in the design of therapeutic drugs.

        Currently, the phenotype is determined by measurements of the molar ratio of metabolites of the drug or a probe drug in the urine samples by high pressure liquid chromatography (HPLC) or capillary electrophoresis (CE), hence using methods which are not readily available in a clinical laboratory.

Drugs metabolized by metabolic enzymes of patent

        The enzymes NAT1, CYP1A2, CYP2D6, CYP2E and CYP 3A4 are involved in the metabolism of large number of drugs. Table 1 lists the wide array of medications that are metabolized and the enzymes involved. These include drugs used for a variety of diseases, including asthma (theophylline), malaria (dapsone), breast cancer (tamoxifen), cardiovascular disease (procainimide), organ transplant (cyclosporine), common medications such as painkillers (acetaminophen, codeine), general anesthetics (lidocaine), and anxiolitics (valium). The wide array of medications to which screening is applicable with these enzymes, demonstrates the potential and the impact that a rapid phenotype

screening can have on the outcome and safety of a
patient's treatment.

Table 1

Drugs metabolized by xenobiotic enzymes phenotyped by
CMPD

Enzyme	Drug
NAT1	p-aminobenzoic acid, p-aminosalicylic acid, dapsone
CYP1A2	Caffeine, theophylline, imipramine, proprano-lol, clozapine, 17β-estradiol (sex hormone) , urorporhyrinogen, lidocaine, propafenone, tam-oxifen (antiestrogen)
CYP2D6	**Psychotropic drugs**: amiflamine, amitryptyline, clomipramine, clozapine, desipramine, haloperi-dol, imipramine, maprotiline, methoxyphenamine, minaprine, nortriptyline, paroxetine, perphena-zine, remoxipride, thioridazine, tomoxetine, trifluperidol, zuclopenthixol. **Cardiovascular agents:** bufuralol, debrisoquine, encainide, flecainide, guanoxan, indoramin, metoprolol, mexiletin, n-propylajmaline, propa-fenone, propranolol, sparteine, timolol, vera-pamil. **Miscellaneous agents:** chlorpropamide, codeine, dextromethorphan, methamphetamine, perhexilene, phenformin.
CYP2E1	Ethanol, acetone, acetaminophen, nitrosamines, nitrosodimethylamine, p-nitrophenol
CYP3A4	Benzodiazepines, cyclosporin, dextromethorphan dihydropyridines, doxorubicin, erythromycin, etoposide, lidocaine, lovastatin, midazolam, paclitaxel, tamoxifen Calcium Channel Blockers: Nifedipine, Diltia-zem, Verapamil.

Associations of metabolic enzymes with altered cancer susceptibility

The metabolic enzymes are responsible for the metabolism of many carcinogenic compounds. Therefore, alterations in the activity of these enzymes alter the biological activity of many carcinogens. Table 2 lists the xenobiotics that are metabolized by the enzymes.

Table 2
Enzymes and the carcinogens they metabolize

Enzyme	Carcinogen
NAT1	diaminobenzidine, N-hydroxy-4-aminobiphenyl; heterocyclic aromatic amines (MeIQx and PhIP)
NAT2	4-aminobiphenyl, diaminobenzidine, heterocyclic aromatic amines (MeIQx, PhIP)
CYP1A2	4-aminobiphenyl, heterocyclic amines (MeIQx, PhIP), 4-methylnitrosamino-1-(3-pyridyl-1-butanone) (NNK, tobacco smoke product)
CYP2D6	Is involved in the metabolism of many carcinogens, however as yet is not reported as the major metabolizer for any
CYP2E1	nitrosodimethylamine, nitrosopyrrolidone, benzene, carbon tetrachloride, 3-hydroxypyridine (tobacco smoke product) .
CYP3A4	N'-nitrosonornicotine (NNN), 4-methylnitrosamino- 1 -(3- pyridyl- 1 -butanone) (NNK), 5-Methylchrysene, 4,4'-methylene-bis(2-chloroaniline) (tobacco smoke products)

Metabolic enzyme phenotypes associated with cancers

The factors influencing cancer development are multi-factorial and it is difficult to associate a cancer with only one cause. However, current research has linked different metabolic phenotypes with increased risk of certain cancers.

Table 3 lists the metabolic enzymes phenotyped by these enzymes and the cancers with which an altered phenotype is linked to an increased susceptibility.

Table 3

Xenobiotic metabolizing enzymes associated with carcinogenesis

Enzyme	Genotype	Cancer	Comments
NAT1	NAT*10	Colorectal	OR = 1,9; 95% CI = 1.2-3.2
		Bladder	Metabolize benzidine
CYP1A2	Fast + Fast NAT2	Colorectal	35% cases vs. 16% controls
CYP2D6	Fast + Slow NAT2	Hepatocellular	OR = 2.6; 95% CI =1.6-4.
CYP2E1	c2	Gastric	OR = 23.6-25.7
CYP3A4	No studies have correlated altered phenotype with altered cancer susceptibility		

NAT1

The NAT1 gene was for a long time classified as monomorphic. However, it is now suggested that NAT1, like the other N-acetyltransferase gene (NAT2), is polymorphic. NAT1 has two phenotypes of slow and rapid metabolizers (e.g. NAT1*4 vs. NAT1*10 genotypes respectively. Measurement of the NAT1 activity is of clinical interest for the following reasons.

Polymorphism

NAT1 is polymorphic and two metabolic phenotypes can be distinguished: rapid, and slow metabolizers. NAT1 metabolizes several drugs and dietary constituents including p-aminobenzoic acid, p-aminosalicylic acid, and dapsone.

In addition, NAT1 activates environmental pro-carcinogens especially diaminobenzidine, N-hydroxy-4-aminobiphenyl; heterocyclic aromatic amines (MeIQx and PhIP). In one study it has been shown that individuals who have the NAT1*10 allele, and hence are rapid N-acetylators, are at a greater risk for colorectal cancer (OR = 1,9; 95% CI = 1.2-3.2, while in another study they have an increased risk for bladder cancer (metabolize benzidine.

Inter Ethnic Differences

The activity of NAT1 varies broadly in a given population. Slow, and rapid NAT1 phenotypes have been distinguished. The NAT1*10 genotype that is associated with rapid metabolic phenotype was monitored in three different ethnic populations, Indian, Malaysian and Chinese. The frequency of NAT1*10 allele was 17%, 39% and 30% respectively. While the NAT1*4 genotype associated with slow metabolizers had a frequency in the same populations of 50%, 30% and 35% respectively. Therefore, it is reasonable that, in drug metabolism studies, each ethnic group can be studied separately for evidence of polymorphism and its antinode should not be extrapolated from one ethnic population to another.

Dapsone

A classical example of the need for phenotyping in drug dosing is the case of Dapsone. Dapsone is used in the treatment of malaria and is being investigated for the treatment of Pneumocystis carinii pneumonia in AIDS patient. Adverse effects include rash, anemia, methemoglobinemia, agranulocytosis, and hepatic dysfunction. Dapsone is cleared from the body via the NAT1 metabolizing system. A study has shown a correlation between slow acetylation and increased adverse reactions to dapsone. (46% vs. 17% for slow and fast acetylators respectively. For, these reasons, the utility of a reliable phenotyping test is obvious.

Individualized Therapy

It is well known that it is possible to individualize therapy for a large number of drugs (theophylline, digoxin, aminoglycosidases, dapsone etc…). However, individualization of therapy has been extremely slow to develop because the methods used for drug phenotyping involves high pressure liquid chromatography (HPLC) and capillary electrophoresis (CE), which are costly, time consuming, and require expertise not readily applicable in a clinical laboratory.

It would be highly desirable to be provided a method for determining an individuals NAT1 phenotype using a non-toxic drug so as to predict his/her response and side effects profile to a wide range of potentially toxic drugs.

It would be highly desirable to be provided with an enzyme linked immunosorbent assay (ELISA) kit for the NAT1 phenotyping, which could be accomplished on a

routine basis by any technician with a minimum of
training and does not involve complex equipments.

It would be highly desirable to be provided with
an enzyme linked immunosorbent assay (ELISA) kit, which
5   would enable a physician to individualize therapy of
drugs such as dapsone.

CYP 1A2

CYP 1A2 constitutes 15% of the total CYP 450
enzymes in the human liver. Measurement of the CYP 1A2
10 activity is of clinical interest for the following rea-
sons:

Polymorphism

CYP 1A2 may be polymorphic although it remains
to be established firmly. Three metabolic phenotypes
15 can be distinguished: rapid, intermediate and slow
metabolizers. CYP 1A2 metabolizes several drugs and
dietary constituents including acetaminophen, anti
pyrine, 17 β-estradiol, caffeine, cloipramine,
clozapine, flutamide (antiandrogenic), imipramine,
20 paracetamol, phenacetin, tacrine and theophylline.

In addition, CYP 1A2 activates environmental
pro-carcinogens especially heterocyclic amines and
aromatic amines. In one study it has been shown that
individuals who are fast N-acetylators and have high
25 CYP 1A2 activity are at a greater risk for colorectal
cancer (35% of cases vs. 16% of controls, OR=2.79
(P=0.002).

Induction and Inhibition

CYP 1A2 is induced by a number of drugs and
30 environmental factors such as omeprazole, Iansoprasole,
polyaromatic hydrocarbons and cigarette smoke. CYP 1A2

is inhibited by oral contraceptives, ketoconazole, α-
napthoflavone, fluvoxamine (seronine uptake inhibitor),
furafylline.

Inter Ethnic Differences

5   The activity of CYP 1A2 varies broadly (60 to 70
fold) in a given population. Slow, intermediate and
rapid CYP 1A2 phenotypes have been distinguished. The
proportion of these three CYP 1A2 phenotypes varied
between ethnic groups and countries: % of
10   intermediates: 50, 70, 60, >95, 60, 20 in U.S.A.,
African-American, China, Japan, Italy and Australia
respectively. It is reasonable that, in drug
metabolism studies, each ethnic group can be studied
separately for evidence of polymorphism and its
15   antinode should not be extrapolated from one ethnic
population to another.

Theophylline

A classical example of the need for phenotyping
in drug dosing is the case of Theophylline.
20   Theophylline is used in the treatment of asthma.
However, theophylline toxicity continues to be a common
clinical problem, and involves life-threatening
cardiovascular and neurological toxicity. Theophylline
is cleared from the body via the CYP 1A2 metabolizing
25   system. Inhibition of CYP 1A2 by quinolone antibiotic
agents or serotonine reuptake inhibitors, may result in
theophyline toxicity. For, theses reasons, the utility
of a reliable phenotyping test is obvious.

Individualized Therapy

30   It is well known that it is possible to
individualize therapy for a large number of drugs

(theophylline, digoxin, aminoglycosidases, etc.). However, individualization of therapy has been extremely slow to develop because the methods used for drug phenotyping involves high pressure liquid chromatography (HPLC) and capillary electrophoresis (CE), which are costly, time consuming, and require expertise not readily applicable in a clinical laboratory.

It would be highly desirable to be provided a method for determining an individuals CYP 1A2 phenotype using a non-toxic drug so as to predict his/her response and side effects profile to a wide range of potentially toxic drugs.

It would be highly desirable to be provided with an enzyme linked immunosorbent assay (ELISA) kit for the CYP 1A2 phenotyping, which could be accomplished on a routine basis by any technician with a minimum of training and does not involve complex equipments.

It would be highly desirable to be provided with an enzyme linked immunosorbent assay (ELISA) kit, which would enable a physician to individualize therapy of drugs such as theophylline, tamoxifen or clozapine.

CYP 2D6

CYP 2D6 constitutes 1-3% of the total CYP 450 enzymes in the human liver. Measurement of the CYP 2D6 activity is of clinical interest for the following reasons:

Polymorphism

CYP 2D6 was the first P450 enzyme to demonstrate polymorphic expression in humans. Three metabolic phenotypes can be distinguished: poor, PM, extensive (EM) and ultraextensive (UEM) phenotypes. CYP 2D6

metabolizes a large variety of drugs and dietary
constituents including:

Psychotropic drugs:

amiflamine, amitryptyline, clomipramine, clozapine,
desipramine, haloperidol, imipramine, maprotiline,
methoxyphenamine, minaprine, nortriptyline, paroxetine,
perphenazine, remoxipride, thioridazine, tomoxetine,
trifluperidol, zuclopenthixol.

Cardiovascular agents:

bufuralol, debrisoquine, encainide, flecainide,
guanoxan, indoramin, metoprolol, mexiletin, n-
propylamaline, propafenone, propranolol, sparteine,
timolol, verapamil.

Miscellaneous agents:

chlorpropamide, codeine, dextromethorphan, methamphet-
amine, perhexilene, phenformin.

In addition, CYP 2D6 is involved in the
metabolism of many carcinogens, however as yet is not
reported as the major metabolizer for any. In one study
it has been shown that individuals who are fast CYP 2D6
metabolizers and slow N-acetylators are at a greater
risk for hepatocellular cancer (OR = 2.6; 95% CI =1.6-
4.

Induction and Inhibition

CYP 2D6 is inhibited in vitro by quinidine and
by viral protease inhibitors as well as by appetite
suppressant drugs such as D- and L-fenfluramine.

Inter Ethnic Differences

The activity of CYP 2D6 varies broadly in a
given population. Poor (PM), extensive (EM) and
ultraextensive (UEM) phenotypes of CYP 2D6 have been
distinguished. The PCYP 2D6 gene is inherited as an

autosomal recessive trait and separates 90 and 10% of
the white European and North American population into
extensive (EM) and poor (PM) metabolizer phenotypes
respectively. In another study the percentage of PM in
different ethnic populations was observed, and white
North Americans and Europeans have 5-10% PM's, American
blacks, 1.8%, Native Thais, 1.2%, Chinese 1%, Native
Malay population, 2.1%, while the PM phenotype appears
to be completely absent in the Japanese population.

It is reasonable that, in drug metabolism
studies, each ethnic group can be studied separately
for evidence of polymorphism and its antinode should
not be extrapolated from one ethnic population to
another.

Dextromethorphan/ Antidepressants

An example of the need for phenotyping in drug
dosing is the case of dextromethorphan.
Dextromethorphan is a nonopioid antitussive with
psychotropic effects. However, Dextromethorphan doses
range from 0 to 6 mg/kg based on individual subject
tolerance. Dextromethorphan is activated via the CYP
2D6 metabolizing system. Dextromethorphan produced
qualitatively and quantitatively different objective
and subjective effects in poor vs. extensive
metabolizers (mean performance +/- SE, 95+/-0.5% for
EMs vs. 86+/-6% for PMs; $p < 0.05$.

Another important drug for CYP 2D6 phenotyping
is the tricyclic antidepressants. For both the PM and
UEM phenotypes of CYP2D6 are at risk of adverse
reactions. PM individuals given standard doses of these
drugs will develop toxic plasma concentrations,
potentially leading to unpleasant side effects

including dry mouth, hypotension, sedation, tremor, or in some cases life-threatening cardiotoxicity. Conversely, administration of these drugs to UEM individuals may result in therapeutic failure because
5   plasma concentrations of active drugs at standard doses are far too low. For, these reasons, the utility of a reliable phenotyping test is obvious.

Individualized Therapy

       It is well known that it is possible to
10 individualize therapy for a large number of drugs (theophylline, digoxin, aminoglycosidases, dextramethorphan etc.). However, individualization of therapy has been extremely slow to develop because the methods used for drug phenotyping involves high
15 pressure liquid chromatography (HPLC) and capillary electrophoresis (CE), which are costly, time consuming, and require expertise not readily applicable in a clinical laboratory.

       It would be highly desirable to be provided a
20 method for determining an individuals CYP 2D6 phenotype using a non-toxic drug so as to predict his/her response and side effects profile to a wide range of potentially toxic drugs.

       It would be highly desirable to be provided with
25 an enzyme linked immunosorbent assay (ELISA) kit for the CYP 2D6 phenotyping, which could be accomplished on a routine basis by any technician with a minimum of training and does not involve complex equipments.

       It would be highly desirable to be provided with
30 an enzyme linked immunosorbent assay (ELISA) kit, which would enable a physician to individualize therapy of drugs such as dextramethorphan, clozapine or verapamil.

CYP 2E1

CYP 2E1 constitutes approximately 5% of the total CYP 450 enzymes in the human liver. Measurement of the CYP 2E1 activity is of clinical interest for the following reasons:

Polymorphism

There is some evidence of genetic polymorphism of CYP 2E1 in the human population, however, the molecular mechanisms remain to be further characterized. Studies have demonstrated the presence of two alleles, designated c1 and c2. Initial studies have shown a possible linkage of c2 allele to higher CYP 2E1 expression.

CYP 2E1 metabolizes several drugs and dietary constituents including ethanol, acetone, acetaminophen, nitrosamines, nitrosodimethylamine, p-nitrophenol.

In addition, CYP 2E1 activates environmental pro-carcinogens especially nitrosodimethylamine, nitrosopyrrolidone, benzene, carbon tetrachloride, 3-hydroxypyridine (tobacco smoke product). In one study it has been shown that individuals who have high CYP 2E1 (c2) activity are at a greater risk for gastric cancer (OR = 23.6-25.7.

Induction and Inhibition

CYP 2E1 is induced by a number of drugs and environmental factors such as cigarette smoke as well as by starvation and in uncontrolled diabetes. CYP 2E1 is inhibited by chlormethiazole, trans-1,2-dichloroethylene and by the isoflavonoids genistein and equol.

Inter Ethnic Differences

The proportion of CYP 2E1 phenotypes varied between ethnic groups and countries: The frequency of the rare c2 allele is about 4% in Caucasians and 20% in the Japanese and a study of a separate polymorphism described a rare C allele that has a frequency of about 10% in Caucasian and 25% in Japanese population. In one study it was shown that Japanese males had much lower levels of CYP2E1 activity as compared to Caucasian males. In another study, it was demonstrated that a Nicaraguan population of mixed white (Spanish) and Asian (central American Indians) origins have an intermediate level of CYP 1A2 allele mutations as compared to the parent populations. Therefore, it is reasonable that, in drug metabolism studies, each ethnic group can be studied separately for evidence of polymorphism and its antinode should not be extrapolated from one ethnic population to another.

Acetaminophen

An example of the need for phenotyping in drug dosing is the case of acetaminophen. Acetaminophen is a widely used painkiller. However, acetaminophen causes hepatotoxicity at low frequency. The hepatotoxicity is due to its transformation via CYP 2E1, to a reactive metabolite (N-acetyl-p-benzoquinoneimine) which is capable of binding to nucleophiles. For, these reasons, the utility of a reliable phenotyping test is obvious.

Individualized Therapy

It is well known that it is possible to individualize therapy for a large number of drugs (theophylline, digoxin, aminoglycosidases,

acetaminophen etc…). However, individualization of therapy has been extremely slow to develop because the methods used for drug phenotyping involves high pressure liquid chromatography (HPLC) and capillary electrophoresis (CE), which are costly, time consuming, and require expertise not readily applicable in a clinical laboratory.

It would be highly desirable to be provided a method for determining an individuals CYP 2E1 phenotype using a non-toxic drug so as to predict his/her response and side effects profile to a wide range of potentially toxic drugs.

It would be highly desirable to be provided with an enzyme linked immunosorbent assay (ELISA) kit for the CYP 2E1 phenotyping, which could be accomplished on a routine basis by any technician with a minimum of training and does not involve complex equipments.

It would be highly desirable to be provided with an enzyme linked immunosorbent assay (ELISA) kit, which would enable a physician to individualize therapy of drugs such as acetaminophen.

CYP 3A4

The CYP 3A family constitutes approximately 25% of the total CYP 450 enzymes in the human liver. Measurement of the CYP 3A4 activity is of clinical interest for the following reasons:

Polymorphism

A large degree of interindividual variability in the expression of the CYP 3A4 isoenzymes has been shown in the human liver (>20 fold) however, no genetic basis for this polymorphic expression has been defined to date. CYP 3A4 metabolizes several drugs and dietary

constituents including benzodiazepines, erythromycin, dextromethorphan dihydropyridines, cyclosporin, lidocaine, midazolam, nifedipine, terfenadine cyclosporine A.

5   In addition, CYP 3A4 activates environmental pro-carcinogens especially N'-nitrosonornicotine (NNN), 4-methylnitrosamino- 1 - (3- pyridyl- 1 -butanone) (NNK), 5-Methylchrysene, 4,4'-methylene-bis(2-chloroaniline) (tobacco smoke products).

10 **Induction and Inhibition**

CYP 3A4 is induced by a number of drugs such as dexamethasone, phenobarbital, primidone and the antibiotic rifampicin. Conversely CYP 3A4 is inhibited by erythromycin, grapefruit juice, indinavir, 15 ketoconazole, miconazole, quinine, and saquinavir.

Cyclosporine

An example of the need for phenotyping in drug dosing is the case of cyclosporine in the treatment of organ transplant patients. Cyclosporine is an 20 immunosuppressant administered post transplant to protect the new organ from being rejected. Plasma levels of this drug are critical as high levels lead to renal toxicity but low levels can lead to organ rejection. Cyclosporine is metabolized via the CYP 3A4 25 system. Several studies have indicated the importance of monitoring CYP 3A4 activity in maintaining an effective and safe cyclosporine dose. For, these reasons, the utility of a reliable phenotyping test is obvious.

30 **Individualized Therapy**

It is well known that it is possible to individualize therapy for a large number of drugs

(theophylline, digoxin, aminoglycosidases, cyclosporine etc.). However, individualization of therapy has been extremely slow to develop because the methods used for drug phenotyping involves high pressure liquid chromatography (HPLC) and capillary electrophoresis (CE), which are costly, time consuming, and require expertise not readily applicable in a clinical laboratory.

It would be highly desirable to be provided a method for determining an individuals CYP 3A4 phenotype using a non-toxic drug so as to predict his/her response and side effects profile to a wide range of potentially toxic drugs.

It would be highly desirable to be provided with an enzyme linked immunosorbent assay (ELISA) kit for the CYP 3A4 phenotyping, which could be accomplished on a routine basis by any technician with a minimum of training and does not involve complex equipments.

It would be highly desirable to be provided with an enzyme linked immunosorbent assay (ELISA) kit, which would enable a physician to individualize therapy of drugs such as cyclosporine.

SUMMARY OF THE INVENTION

One aim of the present invention is to provide an enzyme linked immunosorbent assay (ELISA) kit for the rapid determination of metabolic enzyme phenotype, which can be used on a routine basis in a clinical laboratory.

Another aim of the present invention is to provide an ELISA kit which allows a physician to:

a)   individualize therapy of drugs metabolized by these enzymes

b)   to predict susceptibility to carcinogen induced diseases such as various cancers.

Another aim of the present invention is to provide a method for determining an individual's metabolic enzyme phenotype using a non-toxic drug so as to predict his/her response and side effects profile to a wide range of potentially toxic drugs.

The ELISA phenotyping kits will use non-toxic probe drugs for the determination of the individuals spectrum of metabolic enzyme phenotypes. Table 4 lists the probe drugs that are to be used for each of the proposed enzymes.

Table 4

Enzymes and probes drugs

Enzyme	Probe Drug
NAT1	p-aminosalicylic acid
CYP1A2	Caffeine
CYP2D6	Dextromethorphan
CYP2E1	Chlorzoxazone
CYP3A4	Dextromethorphan

These drugs are consumed by the individual to be phenotyped, and the individuals urine collected 4 hours after consumption. The urine will be analyzed via the ELISA technology developed in the present invention. The urine samples will be monitored for the following probe drug derivatives (Figs. 1-7), and the molar ratios calculated to reveal the individual phenotypes.

In Examples I and II, a detailed description of the probe drug derivatives and the ELISA development

for CYP 1A2 are illustrated. The materials and methods, and the overall general process described for the development of the CYP1A2 ELISA kit for metabolic phenotyping can be and will be applied to the development of the metabolic phenotyping ELISA kits for NAT1, CYP2D6, CYP2E1 and CYP3A4.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 illustrates p-aminosalicylic acid derivatives for NAT1 phenotyping by ELISA;

Fig. 2 illustrates caffeine derivatives for CYP1A2 phenotyping by ELISA;

Fig. 3 illustrates 1,7dimethylxanthine derivatives for CYP1A2 phenotyping by ELISA;

Fig. 4 illustrates 1,7dimethyluric acid derivatives for CYP1A2 phenotyping by ELISA;

Fig. 5 illustrates dextromethorphan derivatives for CYP2D6 phenotyping by ELISA;

Fig. 6 illustrates chlorzoxazone derivatives for CYP2E1 phenotyping by ELISA;

Fig. 7 illustrates dextromethorphan derivatives for CYP3A4 phenotyping by ELISA;

Fig. 8 illustrates the synthetic routes for the production of caffeine and 1,7-dimethylxanthine derivatives for CYP1A2 phenotyping in accordance with one embodiment of the present invention;

Fig. 9 illustrates the synthetic routes for the production of caffeine and 1,7-dimethyluric acid derivatives for CYP1A2 phenotyping in accordance with one embodiment of the present invention; and

Fig. 10 illustrates a pattern of samples to be pipetted in a Falcon 96-well microtest tissue culture plate.

DETAILED DESCRIPTION OF THE INVENTION

Different probe drugs can be used to determine the CYP 1A2 phenotype (caffeine, theophylline) In accordance with the present invention suitable probe

5   drugs include with out limitation, caffeine, theophylline or acetaminophen.

Of these caffeine is the preferred probe. Caffeine is widely consumed and relatively safe. In previous studies the phenotype has been generally

10 determined from the ratios of 1,7-dimethylxanthine (1,7 DMX) + 1,7-dimethyluric acid (1,7 DMU) and 1,3,7-trimethylxanthine (1,3,7 TMX, caffeine). In these studies, the subjects are given an oral dose of a caffeine containing-substance, and the urinary

15 concentrations of the target metabolites determined by HPLC (Kilbane, A. J. et al. (1990) Clin. Pharmacol. Ther **47**: 470-477; Tang, B.-K. et al. (1991) Clin. Pharmacol. Ther **49**: 648-657) or CE (Meachers et al. (1998) Biomarkers **3**: 205-218).

20   Inhibition of CYP 1A2 by quinolone antibiotic agents or serotonine reuptake inhibitors, may result in theophyline toxicity. For, theses reasons, the utility of a reliable phenotyping test is obvious.

Enzyme linked immunosorbent assays (ELISA) have

25 been successfully applied in the determination of low amounts of drugs and other antigenic compounds in plasma and urine samples and are simple to carry out. We have previously developed an ELISA for N-acetyltransferase-2 (NAT2) phenotyping using caffeine

30 as a probe drug (Wong, P., Leyland-Jones, B., and Wainer, I.W. (1995) J. Pharm. Biomed. Anal. **13**: 1079-1086). We have subsequently tested and proven the

validity of the ELISA for the NAT2 phenotyping
(Leyland-Jones et al. (1999) Amer. Assoc. Cancer Res.
40: Abstract 356). The ELISA for NAT2 phenotyping is
simpler to carry out than the HPLC and CE.

5   In accordance with the present invention, there
are currently being developed antibodies to measure the
molar ratio of caffeine and two caffeine metabolites
(1,7-dimethylxanthine (1,7 DMX), 1,7-dimethyluric acid
(1,7 DMU)) in urine samples of an individual collected

10   after caffeine consumption. This ratio provides a
determination of an individual's CYP 1A2 phenotype.
Subsequently, there will be an antigen enzyme linked
immunosorbent assay (ELISA) for measuring this ratio
using these antibodies. The antibodies of the present

15   invention can be polyclonal or monoclonal antibodies
raised against caffeine and two different metabolites
of caffeine, which allow the measurement of the molar
ratio of caffeine and these metabolites.

          In accordance with the present invention, the

20   molar ratio of caffeine metabolites is used to
determine the CYP 1A2 phenotype of the individual as
follows:

$$\frac{1,7\text{-dimethylxanthine (1,7 DMX)} + 1,7\text{-dimethyluric acid (1,7 DMU)}}{\text{caffeine}}$$

25   Molar ratios of 4 and 12 separate slow,
intermediate and fast CYP 1A2 metabolizers (Butler et
al. (1992) Pharmacogenetics **2:** 116-117).

MATERIALS AND METHODS

30   **Materials**

          N-acetyl-p-aminophenol (acetaminophen), dioxane,
formic acid 98-100 % glass redistilled and isobutyl

chloroformate are purchased from A&C American Chemicals
Ltd. (Ville St-Laurent, Que. Canada); horse radish
peroxidase is purchased from Boehringer Mannheim
(Montreal, Que., Canada); ELISA plates (96-well Easy
Wash™ modified flat bottom, high binding; Corning glass
wares, Corning, NY, USA) and Falcon 96-well microtest
tissue culture plate, no. 3072 (Beckton Dickinson
Labware, Franklin, NJ, USA) are purchased from Fisher
(Montreal, Quebec, Canada); alkaline phosphatase
conjugated to goat anti-rabbit IgGs, Keyhole limpet
hemocyanin (KLH) is from Pierce Chemical Co. (Rockford,
IL, USA); acetic anhydride, acetonitrile HPLC grade,
benzylurea, bovine serum albumin (Cat. No A-3803), N-
bromosuccinimide, caffeine metabolites; 1-ethyl-3-(3-
dimethylaminopropyl) carbodiimide hydrochloride
solution (EDAC), ethyl 4-bromobutyrate, ethyl 6-bro-
mohexanoate, methyl cyanoacetate, deuterated chloroform
(CDCl$_3$) , deuterated dimethylsulfoxide (d$_6$), deuterated
oxide (D$_2$O), 1,4-diaminobutane, diethanolamine,
dimethylformamide, dimethylsulfate, di-tert-butyl
dicarbonate, ethyl chloroformate, Freund's adjuvant
(complete and incomplete), glutaraldehyde (50 % v/v),
1-methylxanthine, p-nitrophenolphosphate disodium salt,
palladium, 10 wt. % (dry basis) on activated carbon, o-
phenylenediamine hydrochloride, polyoxyethylene
sorbitan monolaurate (Tween 20), porcine skin gelatin,
protein A-Sepharose 4B, Sephadex ™ G25 fine, sodium
hydride, sodium methoxide, theophylline, tributylamine,
Tween ™ 20, are purchased from Sigma-Aldrich (St-
Louis, Missouri, USA); Silica gel particle size 0.040-
0.063 mm (230-400 mesh) ASTM Emerck Darmstadt, Germany
was purchased from VWR (Montreal, Que., Canada).

Dioxane is dried by refluxing over calcium hydride for 4 hours and distilled before use. Other reagents were ACS grade.

Synthetic procedures

5   The synthetic routes for the production of caffeine, 1,7-dimethylxanthine, 1,7-dimethyluric acid derivatives are shown in Figs. 8 and 9.

Synthesis of 7-ethoxycarboxypentyl-1,3-dimethylxanthine (II)

10   Compound II is synthesized by a procedure similar to that of Daly et al. (Daly, J.W., Mueller, C., Shamin, M. (1991) Pharmacology, 42: 309-321). 320 mg of theophylline (I) (1.78 mmole) is dissolved in 7 mL of dry dimethylformamide and 290 mg of potassium 15 carbonate (2.1 mmole) is added to the reaction mixture. 358 µL of ethyl 6-bromohexanoate (2.02 mmole) is slowly added and the suspension is heated at 60°C for 14 hours. The suspension is filtered in order to remove the potassium carbonate. After washing the potassium 20 carbonate with some dimethylformamide, the solvent is evaporated under reduced pressure with a rotary evaporator and a high vacuum pump. The residue is dissolved in chloroform and the solution is dried over magnesium sulfate ($MgSO_4$). The solvent is evaporated 25 under reduced pressure with a rotary evaporator. 480 mg of the product (slightly yellow oil 1.49 mmole) is obtained, corresponding to a yield of 83.7%.

Synthesis of 7-carboxypentyl-1,3-dimethylxanthine (III)

      Compound III is synthesized as follows. 225 mg 30 of compound II (0.7 mmole) is dissolved in 7 mL of dimethylformamide. 4 mL of a 10% NaOH solution is added and the solution is refluxed for 30 min (100-125 °C).

The solvents are evaporated under reduced pressure with a rotary evaporator and a high vacuum pump. The residue is dissolved in 7 mL of water and the solution is acidified to pH 4 with a 6N HCl solution. Cooling the solution at 4^0 C crystallizes the product as needle-like crystals. The crystals are filtered under vacuum trough a 15-mL sintered glass funnel (10-15 ASTM) and dried. 175 mg of the product is obtained (0.595 mmole), corresponding to a yield of 85%.

Synthesis of 7-ethoxycarboxylpentyl-1-methylxanthine (V)

Compound V is synthesized as follows. 116 mg of 1-methyxanthine (IV) (0.7 mmole) is dissolved in 4 mL of dimethylformamide. 129 mg of potassium carbonate (0.93 mmole) is added and the resulting solution is stirred. 125 µL of ethyl-6-bromohexanoate (0.7 mmole) in 0.4 mL dimethylformamide is slowly added in three portions. The reaction mixture is heated at 50 ^{0}C for 1.5 hours and at 65 ^{0}C for 1 hour. After cooling, the suspension is filtered and the filtrate is evaporated under reduced pressure with a rotary evaporator and a high vacuum pump. The product is purified by flash chromatography on a silica gel column (40 x 1 cm) using an ethyl acetate-hexane solution (9:1, v/v) as the eluent.

Synthesis of 7-carboxypentyl-1-methylxanthine (VI)

Compound VI is synthesized as follows. 31 mg of compound V (0.1 mmol) is dissolved in 1 mL of dimethyl-formamide and 660 µL of a 10% NaOH is added. The resulting solution is refluxed for 30 min (100-120 ^{0}C). After cooling at room temperature, the solvent is

evaporated under reduced pressure with a rotary evaporator and a high vacuum pump. The residue is dissolved in water and acidified to pH 4 with a 6N HCl solution. Upon cooling, the solution yields white
5 needle-like crystals, which are filtered and dried. 23 mg of the product (0.082 mmole) is obtained, corresponding to a yield of 82%.

Synthesis of 6-amino-1-benzyl uracil (IX)

Compound IX is synthesized according to the
10 procedure similar of that of Hutzenlaub and Pfeiderer (Hutzenlaub, W., and Pfeiderer, W. (1979). Liebigs Ann. Chem. 1847-1854) as follows. 8.64g of sodium methoxide (160 mmol) is dissolved in 71mL methanol. The solution is stirred and 7.55g of benzylurea (50 mmol) and 4.71mL
15 methyl cyanoacetate (53.4 mmol) are added. The suspension is refluxed 5.5 hours at 68-70°C and cooled at room temperature. After filtration, the methanol is evaporated under reduced pressure with a rotary evaporator. The residue is dissolved in warm distilled
20 water, and the product is precipitated by acidification to pH 3-4 with glacial acetic acid. After 2 hours (or overnight) at room temperature, the suspension is filtered under vacuum through a sintered glass funnel. The product is washed with water and dried. The yield
25 is 62-65%.

Synthesis of 6-amino-1-benzyl-5-bromouracil (X)

Compound X is synthesized according to the procedure of Hutzenlaub and Pfeiderer (Hutzenlaub, W., and Pfeiderer, W. (1979). Liebigs Ann. Chem. 1847-1854)
30 as follows. 3.2g of 6-amino-1-benzyluracil (15.8 mmol) is dissolved at 100° C in 60 mL acetic acid and 3 mL

acetic anhydride. 2.85 g of N-bromosuccinimide (16 mmol) is added in small portions over the next 30 minutes. The reaction mixture is stirred for 1 hour and cooled at room temperature. The precipitate is filtered and washed with small amount of cold ethanol and dried. 3.36 g of white crystals are obtained (12 mmol), corresponding to a yield of 76%.

Synthesis of 6-amino-1-benzyl-5-[N-4'-aminobutyl)-amino] uracil (XI)

Compound XI is synthesized as follows. 3g of compound X (10.71 mmol) is dissolved in 30 mL of 50% 1,4-diaminobutane (bp 158-160°; d 0.877) in water (v/v) and the solution is stirred overnight at room temperature. The solution is evaporated under reduced pressure with a rotary evaporator and a high vacuum pump. The resulting oil is dissolved in a minimal amount of ethyl acetate-methanol solution (4:1; v/v) and is purified by dry flash chromatography on a silica gel packed in a sintered glass funnel (150 mL) with ethyl acetate-methanol solutions as the eluents. At each successive fraction, the solvent polarity was increased, varying from 60% ethylacetate/40% methanol to 45% ethylacetate/55% methanol (v/v). The product is isolated as a light yellow oil. The amount of purified product obtained is 1.69g (6.1 mmol), corresponding to a yield of 57%.

Synthesis of 6-amino-1-benzyl-5-[N-4'-tert-butoxycar-bonyl-amino]uracil (XII)

Compound XII is synthesized as follows. 1.63g of compound XI (5.9 mmol) is dissolved in 5.4 mL of 1 N NaOH solution. 270 mg of sodium bicarbonate (3.2 mmol) and 2.7 mL of water are added. 5.4 mL of di-tert-butyl dicarbonate solution in isopropanol (1.88g (8.61 mmol)

is dissolved in 5.4 mL isopropanol) is added slowly to
the solution of compound XI. After stirring for 3 hours
at room temperature, 13.4 mL of water is added and the
unreacted di-tert-butyl dicarbonate is extracted twice

5   with 20mL of petroleum ether. The pH of the reaction
mixture is adjusted to 7 by the addition of a 10%
citric acid solution and the solution is extracted
twice with 40mL ethyl acetate. The organic layer is
dried over sodium sulfate (Na_2SO_4) and is concentrated

10   under reduced pressure with a rotary evaporator. The
product is precipitated by the addition of some light
petroleum ether to the concentrated solution. 0.99 g of
an off-white crystalline compound XII (2.62 mmol) is
obtained corresponding to a yield of 44%.

15   **Synthesis of 6-amino -1- benzyl-5-[(N-4'tert-butoxy-
carbonylaminobutyl-N-ethoxycarbonyl)-amino]-uracil
(XIII)**
        Compound XIII is synthesized as follows. 806 mg
of compound XII (2.14 mmol) was suspended in 7.5 mL of

20   water and stirred energetically. 0.5 mL of ethyl
chloroformate (5.22 mmol) is added. 3.75 mL of a 1N
NaOH solution is added drop wise and the resulting
solution is stirred at room temperature for 2.5 hours.
The white solid product is filtered, washed thoroughly

25   with water and dried. 741 mg of the product is obtained
(1.77 mmol), corresponding to a yield of 82.7%.

**Synthesis   of   6-amino-1-benzyl-5-[(N-4'tert-
butoxycarbon-ylaminobutyl-N-ethoxycarbonyl)-amino]-3-
methyluracil(XIV)**

30   Compound XIV is synthesized as follows. 712 mg
of compound XIII (1.77 mmol) is suspended is 5.8mL of
water. 2.3mL of a 1N NaOH solution are added and the
resulting solution is heated at 40⁰ C and vigorously
stirred. 0.23mL dimethylsulfate (2.43 mmol) is slowly

added and the resulting solution stirred at 40 ° C for
1.5 hours. The precipitate, which formed during the
reaction, is filtered, washed with water and dried. The
product is purified from the precipitate by flash
5   chromatography on a silica gel column (40 x 1cm) using
a solution of 4% methanol in dichloromethane as eluent.
The product is recrystallized from ethyl acetate. 498
mg of compound XIV (1.15 mmol) is obtained,
corresponding to a yield of 65%.

10   **Synthesis of 6-amino-5-[(N-4'tert-butoxycarbonylamino-
butyl-N-ethoxycarbonyl)-amino]-3-methyluracil (XV)**
          Compound XV is synthesized as follows. 440mg of
compound XIV (1.02 mmol) is dissolved in 12 mL methanol
and mixed with 252mg ammonium formate (4 mmol). 240mg
15   of palladium-on-charcoal (10%) are added under nitrogen
atmosphere. The catalytic hydrogenation is performed at
room temperature for 3 hours. The catalyst is removed
by filtration and the filtrate is evaporated under
reduced pressure with a rotary evaporator and a high
20   vacuum pump. 341 mg of the product is obtained (0.99
mmol) corresponding to a yield of 97%.

Synthesis of 7-(4' aminobutyl)-1-methyluric acid (XVI)

          Compound XVI is synthesized as follows. 300mg of
compound XV (0.875 mmol) is dissolved in 4.5mL dry
25   dimethylformamide and mixed with 144 mg of sodium
hydride (6 mmol). The mixture is stirred at room
temperature for 20 min and at 110-115 °C for 30 min. The
color changes slowly to a dark yellow. After cooling,
6.5mL of water are added and the solution is acidified
30   to pH 0 with a 6N HCl solution. The solvents are
evaporated under reduced pressure with a rotary
evaporator and a high vacuum pump, and the crude

product is dissolved in a ethyl acetate-methanol solution (1:4, v/v). The inorganic salt is removed by filtration and the yellow filtrate is purified by flash chromatography on a silica gel column (40 x 1 cm) using

5   a solution of ethyl acetate-methanol (3:7, v/v) as the eluent. The fraction containing the pure product was evaporated under reduced pressure with a rotary evaporator. After titration of the residue with isopropanol, the product is obtained as a pale yellow

10 solid. 98.9 mg of the product is obtained (0.391 mmol) corresponding to a yield of 45%.

NMR Spectroscopy

^{1}H NMR spectra of synthesized were obtained using a 500 mHz spectrophotometer (Varian XL 500 mHz,

15 Varian Analytical Instruments, San Fernando, CA, USA).

Conjugation of haptens to bovine serum albumin and keyhole limpet hemocyanin

Caffeine-BSA, 1,7-Dimethylanthine-BSA conjugates are prepared by procedure similar to that of Rojo et

20 al. (Rojo et al. (1986) J Immunol. **137**: 904-910). Fifteen mg of BSA is dissolved in 6 mL of a caffeine derivative (or 1,7-dimethylxanthine derivative) solution (1.25 µmoles/mL of water) in a 25-mL erlenmeyer flask followed by the addition of 1.43 mL of

25 an EDAC solution (10 mg/mL of water). The solution is stirred overnight at room temperature and dialyzed against 500 mL water at room temperature for 48 h with two changes per day of the water. The conjugates are stored as 0.5 mL-aliquots at -20^0 C. The 1,7-

30 Dimethyluric acid conjugate is prepared by the method of Peskar et al. (Peskar (1972) Eur. J. Biochem. **26**:

191-195). 7.5 mg of 1,7 dimethyluric acid (0.03 mmole) is placed in a 5 mL round bottom flask and is dissolved with 1 mL of a 0.1M $Na_2PO_4-NaH_2PO_4$ buffer, pH 7.0. A volume of 500 µL of a 0.021 M glutaraldehyde solution

5   (42.5 µL 50 % glutaraldehyde (v/v) per 10 mL of water) is added to the stirred solution. After stirring for 2 hours, 100 µL of a 1M lysine in 0.1M $Na_2PO_4-NaH_2PO_4$ buffer, pH 7.0 is added. The solution is stirred for one hour and dialyzed against 250 mL of a 150 mM NaCl,

10   5 mM $Na_2PO_4-NaH_2PO_4$ buffer, pH 7.0 for 48 hours with 2-3 changes per day of the buffer. Solution of 1,7-dimethyluric acid-BSA conjugate was stored as 0.5 mL aliquots at -20^0 C. Caffeine-KLH and 1,7-dimethylxanthine-KLH conjugates are prepared as

15   follows. 20 mg of lyophilized powder of KLH is dissolved with 2 mL of a 0.9 M NaCl solution and dialyzed against 100 mL for 10 hours with 2 changes of the solution. To 1.1 mL KLH solution (approximately 10 mg/mL) in a 25-mL erlenmeyer flask, is added 0.8 mL of

20   the caffeine derivative or the 1.7-dimethylxanthine derivative (2.5 µmol/mL of a 0.9 M NaCl). 2 mL of an EDAC solution (10 mg/mL of 0.9 M NaCl), and 1.8 mL 0.9 M NaCl solution are successively added to the derivative solution. The solution is stirred overnight

25   (20 hours) at room temperature. The solution is dialyzed against 250 mL of a 0.9 M NaCl solution for 48 hours with 2-3 changes of the solution per day. The caffeine-KLH and 1.7-dimethylxanthine-KLH solutions are stored as 0.5 mL aliquots at -20^0 C. The 1,7-

30   dimethyluric acid-KLH conjugate is prepared according to a method similar to that of Peskar et al. (Peskar

(1972) Eur. J. Biochem. **26:** 191-195). 20 mg of lyophilized powder of KLH is dissolved with 2 mL of a 0.9 M NaCl solution and dialyzed against 100 mL for 10 hours with 2 changes of the solution. 7.3 mg of 1,7 dimethyluric acid (approximately 0.03 mmole) is placed in a 5 mL round bottom flask and is dissolved with 1 mL of a KLH solution A volume of 500 µL of a 0.021 M glutaraldehyde solution (42.5 µL 50 % glutaraldehyde (v/v) per 10 mL of water) is added dropwise to the stirred solution. After stirring for 2 hours, 100 µL of a 1M lysine in 0. 1M Na_2PO_4-NaH_2PO_4 buffer, pH 7.0 is added. The solution is stirred for one hour and dialyzed against 250 mL of a 0.9M NaCl, 5 mM Na_2PO_4-NaH_2PO_4 buffer, pH 7.0 for 48 hours with 2-3 changes per day of the buffer. Solution of 1,7-dimethyluric acid-BSA conjugate was stored as 0.5 mL aliquots at -20^0 C.

Protein Determination by the method of Lowry et al (Lowry, O.H. et al. (1951) J. Biol. Chem., **193:** 265-275)

Solutions

Solution A: 2g Na_2CO_3 is dissolved in 50 mL water, 10 mL of 10% SDS and 10 mL 1N NaOH, bring to 100 mL volume with water. Freshly prepared.

Solution B: 1% NaK Tartrate

Solution C: 1% $CuSO_4$.5H2O

Solution D: 1N phenol (freshly prepared): 3mL Folin & Ciaocalteu's phenol reagent (2.0 N) and 3 mL water

Solution E: 98 mL Solution A, 1 mL Solution B, 1 mL Solution C. Freshly prepared

BSA: 1 mg/mL. 0.10 g bovine serum albumin (fraction vol.)/100 mL.

Assay

Standard curve   **Tube # (13 x 100mm)**

Solution	1	2	3	4	5	6	7
BSA (µl)	0	10	15	20	30	40	50
Water (µl)	200	190	185	180	170	160	150
Solution E (mL)	2.0	2.0	2.0	2.0	2.0	2.0	2.0

Vortex and leave 10 min at room temperature.

Solution D (µl)   200   200   200   200   200   200   200

Vortex and leave at room temperature for 1 hour.

5   Read absorbance at 750 nm using water as the blank.

Unknown

Solution D.F[a] (in triplicate)   **Tube # (13 x 100 mm)**

Unknown (µl)	x	x	x	
Water (µl)	y	y	y	x + y = 200 µl

10   Solution F (mL)   2.0   2.0   2.0

Vortex and leave 10 min at room temperature.

Solution D (µl)   200   200   200   200   200   200   200

Vortex and leave at room temperature for 1 hour.

Read absorbance at 750 nm using water as the blank.

15   Calculate the protein concentration using the standard curve and taking in to account the D.F. (dilution factor) of the unknown

a: D.F. (dilution factor): has to be such that the absorbance of the unknown at 750 nm is with in the

20   range of absorbance of the standards.

Methods to determine the amounts of moles of caffeine , 1,7-DMX or 1,7-DMU incorporated per mg of KLH

This method gives an approximate estimate. It is useful because it allows the determination of whether

25   the coupling proceeded as expected.

A) Solutions

- 10 % sodium dodecyl sulfate (SDS) solution
- 1 % SDS solution
- 0.5 or 1 mg/mL of caffeine-KLH (or 1,7-DMX-KLH or 1,7-DMU-KLH) in a 1 % SDS solution (1 mL).
5   - 0.5 or 1 mg/mL KLH in a 1 % SDS solution.

B) Procedure

- Measure the absorbance of the caffeine-KLH conjugate (or 1,7-DMX-KLH or 1,7-DMU-KLH) at the wavelength of absorption maximum of caffeine (or 1,7-DMX or 1,7-DMU) with 1 % SDS solution as the blank
- Measure the absorbance of the KLH solution at the wavelength of absorption maximum of caffeine (or 1,7-DMX or 1,7-DMU).
- Calculate the amount of mole of caffeine (or 1,7-DMX or 1,7-DMU) incorporated per mg of KLH with the following formula:

$$y = \frac{A_{\Delta\max}(\text{caffeine} - \text{KLH}) - A_{\Delta\max}(\text{KLH})}{\varepsilon_{\Delta\max}(\text{caffeine})X[\text{KLH}]}$$

20   where:

y is the amount of mole of caffeine/mg of KLH;

$\varepsilon_{\lambda\max}$ (caffeine) is the molar extinction coefficient of caffeine at the wavelength of absorption maximum.

Coupling of haptens to horse radish peroxidase

25   The caffeine and 1,7-dimethylxanthine derivatives and the 1,7-dimethyluric acid derivative (after succinylation with succinic anhydride) were conjugated to horse radish peroxidase (HRP) by the following procedure. Place 0.12 mmol of the derivative 30 in a 5 mL round bottom flask. Pipet 500 μL of dioxane

freshly dried over calcium chloride. Stir the suspension and cool at 10° C in a water bath using crushed ice. Pipet 31 µL isobutylchloroformate (0.24 mmol) (recently opened or purchased) and 114 µL

5 tributylamine (0.47 mmol). Stir for 30 min at 10° C. While stirring, dissolve 13 mg of horse radish peroxidase (HRP) in 2 mL of water and cool the solution at 4° C on crushed ice. After the 30 min. of stirring, pipet 100 µL of a 1N NaOH solution (freshly prepared)

10 at 4° C to the HRP solution and pour the alkaline HRP solution at once in the 5 mL flask. Stir the suspension 4 hours at 10-12° C. Separate the free derivative from the HRP conjugate by filtration on a Sephadex G-25™ fine column (1.6 x 30 cm) equilibrated and eluted with

15 0.1 M sodium phosphate buffer, pH 7.0. Collect fractions of 1.0-1.2 mL manually or with a fraction collector. During elution two bands may be observed: the HRP conjugate and a light yellow band behind the HRP conjugate. The HRP conjugate band eluted between

20 fractions 11-16. Pool fractions containing the HRP conjugate in a 15 mL tissue culture with a screw cap. Determine the HRP conjugate concentration at 403 nm after diluting an aliquot (usually 50 µL + 650 µL of buffer).

25

$$[\text{HRP conjugate}] \ (\text{mg/mL}) = A_{403} \times 0.4 \times \text{D.F.}$$

Record the ultraviolet spectrum (UV) absorption spectrum between 320 and 220 nm. The presence of addi-

30 tional absorption peaks at 280 nm, 280 nm and 290 nm for caffeine-HRP, 1,7-DMX-HRP and 1,7-DMU-HRP

conjugates, respectively, are indicators that the coupling proceeded as expected. After the above measurements, 5 μL of a 4% thiomersal solution is added per mL of caffeine-HRP, 1,7-DMX-HRP or 1,7-DMU-HRP

5 conjugate solution. The conjugates are stored at 4°C.

Antibody Production

Six mature females New Zealand white rabbits (Charles River Canada, St-Constant, Que., Canada) were used for antibody production. The protocol employed in

10 this study was approved by the McGill University Animal Care Committee in accordance with the guidelines from the Canadian Council on Animal Care. An isotonic saline solution (0.6 mL) containing 240 μg of KLH conjugated antigen was emulsified with 0.6 mL of a complete

15 Freund's adjuvant. 0.5 mL of the emulsion (100 μg of antigen) was injected per rabbit intramuscularly or subcutaneously. Rabbits were subsequently boosted at intervals of three weeks with 50 μg of antigen emulsified in incomplete Freund's adjuvant. Blood was

20 collected without anticoagulant in a vacutainer tube by venipuncture of the ear 10-14 days after boosting and kept at 4°C. After clotting, centrifugation at 4°C, sodium azide was added to the antisera to a final concentration of 0.001% (1μL of a 1 % sodium azide

25 solution per mL of antisera). Antisera were stored as 0.5 mL aliquots at -20 °C.

Antiserum titers

The wells of a microtiter plate were coated with 10 μg mL^{-1} of bovine serum albumin-caffeine (or

30 1,7-dimethyl xanthine, 1,7-dimethyluric acid) conjugate in 100 mM sodium carbonate buffer, pH 9.6) overnight at

4° C (150 µL/well). They were then washed three times with TPBS (phosphate buffer saline containing 0.05 % Tween 20) using a Nunc Immuno Wash 12 autoclavable. Unoccupied sites were blocked by an incubation with 150

5 µL/well of TPBS containing 0.05 % porcine gelatin for 2 h at room temperature. The wells were washed three times with TPBS and 150 µL of antiserum diluted in TPBS was added. After 2 h at room temperature, the wells were washed three times with TPBS, and 100 µL of goat

10 anti-rabbit IgGs-alkaline phosphatase conjugate diluted in PBS containing 1% BSA was added. After 1 h at room temperature, the wells were washed three times with TPBS and three times with water. To the wells were added 150 µL of a solution containing $MgCl_2$ (0. 5 mM)

15 and p-nitrophenol phosphate (3.85 mM) in diethanolamine buffer (10 mM, pH 9.8). After 30 min at room temperature, the absorbency was read at 405 nm with a microplate reader. The antibody titer is defined as the dilution required to change the absorbance by one unit

20 (1 au).

Isolation of IgG antibodies

Rabbit IgG antibodies against KLH conjugates were purified by affinity chromatography on a Protein A-Sepharose 4B column as follows. A 0.9 x 15 cm

25 Pharmacia chromatographic column was packed with Protein A-Sepharose 4B suspension to a volume of 1 mL. The column was washed generously with a 0.01 M Na_2HPO_4-NaH_2PO_4 buffer, pH 8.0 containing 0.15M NaCl (PBS) and then washed with 3-4 mL of a 0.1 M trisodium citrate

30 buffer, pH 3.0. The column was then washed generously with PBS. 1 mL of rabbit antiserum is diluted with 1 mL

PBS, and the resulting solution is slowly applied to the column. The column is washed with 15 mL PBS and eluted with a 0.1 M trisodium citrate buffer, pH 3.0. Three fractions of 2.2 mL were collected in 15-mL graduated tubes containing 0.8 mL of 1 M Tris-HCl buffer, pH 8.5. The purified rabbit IgG antibodies were stored at 4 °C in the presence at 0.01 % sodium azide.

Competitive antigen ELISA

Buffers and water without additives are filtered trough 0.45 µM millipore filters and kept for one week, except the substrate buffer which was freshly prepared. BSA, antibodies, Tween™ 20 and horse radish peroxidase are added to buffers and water just prior to use. Urine samples are usually collected four hours after drinking a cup of coffee (instant or brewed with approximately 100 mg of caffeine per cup) and stored at −20°C as 1-mL aliquots in 1.5-mL microtubes. For the ELISA, the urine samples are diluted with isotonic sodium phosphate buffer, pH 7.5 (310 mosM) to give concentrations of caffeine, 1.7-DMX and 1,7-DMU no higher than 3 x 10^{-6} M in the microtiter plate wells. Wells of the ELISA plate were washed with a Nunc-Immuno wash 12 washer. Sixteen mL of a solution of 6.6 µg ml^{-1} of isolated IgG antibodies is prepared in a 100 mM sodium carbonate buffer, pH 9.6, and 150 µL of this solution is pipetted in each well of a microtiter plate using a eight channel pipet (Brinkmann Transferpette™-8 50-200 µL) and 200µL Flex tips from Brinkmann). After coating the wells with antibodies at 4°C for 20 hours, the wells were washed 3 times with the isotonic sodium phosphate buffer containing 0.05% Tween™ 20 (IPBT) and

properly drained by inverting the plate and absorbing
the liquid on piece of paper towel. Thirty mL of a
solution of a IPBT solution containing 1 % BSA is
prepared and 150 µL of this solution is pipetted in
5   each well using a eight channel pipet (Brinkmann
Transferpette™-8 50-200 µL) and 200 µL yellow tips
(Sarstedt yellow tips for P200 Gilson Pipetman). After
3 hours at room temperature, the wells were washed 3
times with IPBT solution and drained. Samples of 400
10   µL for determination of caffeine, 1,7-DMX and 1,7-DMU
are prepared in 1.5-mL microtubes using Sarstedt yellow
tips and a P200 Gilson Pipetman. e) 200 µL of each
sample are pipetted in duplicate in a Falcon 96-well
microtest tissue culture plate according to the pattern
15   shown in Figure 10, using Sarstedt yellow tips and a
P200 Gilson Pipetman. Using an eight channel pipet
(Brinkmann Transferpette™-8 50-200 µL) and changing
the tips of the eight channel pipet (200µL Flex tips
from Brinkmann) at each row, 150 µL of samples are
20   transferred in the corresponding wells of a 96-well
ELISA microtiter plate coated with antibodies. After
the addition of the samples, the microtiter plates are
covered and left standing at room temperature for 2 h.
While the plate is left standing the substrate buffer
25   without the hydrogen peroxide and o-phenylenediamine
hydrochloride is prepared (25 mM citric acid and 50 mM
sodium phosphate dibasic buffer, pH 5.0). The
microtiter plate is washed 3 times with the IPBT
solution and 3 times with a 0.05% Tween™ solution and
30   drained. 50 µL of hydrogen peroxide and 40 mg of o-
phenylenediamine are added to the substrate buffer. One

hundred fifty microliters (150 µL) of the substrate buffer solution is then added to each wells using a eight channel pipet (Brinkmann Transferpette™-8 50-200 µL) and 200µL Flex tips (Brinkmann). The microtiter

5 plate is covered and shaken for 25-30 min at room temperature and the enzymatic reaction is stopped by adding 50 µL/well a 2.5 M HCl solution using an eight channel pipet (Brinkmann Transferpette™-8 50-200 µL) and 200µL Flex tips (Brinkmann). After gently shaking

10 for 3 min., the absorbance is read at 490 nm with a microplate reader.

Standard solutions of Caffeine, 1,7-DMX and 1,7-Dimethyluric acid solutions for ELISA

Prepare a 100 mL stock solution of caffeine, 1,7-DMX

15 and 1,7-DMU acid at concentrations of 6.00×10^{-4} M in the 310 mosM sodium phosphate buffer, pH 7.5 (IPB) in a 100 mL volumetric flask. Stirring the solution to insure complete solubilization.

Store the stock solutions as 1 mL aliquots at –

20 20°C.

On the day of the ELISA, thaw one aliquot and warm up at room temperature.

Prepare the following standard solutions of the above compounds

25

Standard #	[Compound]	Composition
1	6.00×10^{-4} M	Stock solution
2	2.00×10^{-4} M	200 μL S1 + 400 μL IPB
3	1.12×10^{-4} M	200 μL S1 + 868 μL IPB
4	6.00×10^{-5} M	100 μL S1 + 900 μL IPB
5	3.56×10^{-5} M	60 μL S1 + 951 μL IPB
6	2.00×10^{-5} M	100 μL S2 + 900 μL IPB
7	1.12×10^{-5} M	100 μL S3 + 900 μL IPB
8	6.00×10^{-6} M	100 μL S4 + 900 μL IPB
9	3.56×10^{-6} M	100 μL S5 + 900 μL IPB
10	2.00×10^{-6} M	100 μL S6 + 900 μL IPB
11	1.12×10^{-6} M	100 μL S7 + 900 μL IPB
12	6.00×10^{-7} M	100 μL S8 + 900 μL IPB
13	3.56×10^{-7} M	100 μL S9 + 900 μL IPB
14	2.00×10^{-7} M	100 μL S10 + 900 μL IPB
15	1.12×10^{-7} M	100 μL S11 + 900 μL IPB
16	6.00×10^{-8} M	100 μL S12 + 900 μL IPB
17	3.56×10^{-8} M	100 μL S13 + 900 μL IPB
18	2.00×10^{-8} M	100 μL S14 + 900 μL IPB
19	2.00×10^{-9} M	100 μL S15 + 900 μL IPB
20	2.00×10^{-10} M	100 μL S15 + 900 μL IPB
21	2.00×10^{-11} M	100 μL S15 + 900 μL IPB
22	2.00×10^{-12} M	100 μL S15 + 900 μL IPB
23	2.00×10^{-13} M	100 μL S15 + 900 μL IPB

Antibody Specificity

To ensure accuracy in the ELISA measurement of CYP 1A2 phenotyping, the antibodies must have specificity for their individual caffeine metabolites, with little or no recognition of other derivatives. To ensure their selectivity an ELISA will be performed

with standard solutions of the compounds listed in
Table 5. An ideal antibody specificity result is
hypothesized with the Table 5 as well.

Table 5

Cross-reactivity of caffeine-Ab, 1,7-DMX-Ab and 1,7-
DMU-Ab towards caffeine metabolites and structural
analogs

Compound	% Cross-reaction		
	Caffeine-Ab	1,7-DMX-Ab	1,7-DMU-Ab
Caffeine	100	0[a]	0
Xanthine	0	0	0
Hypoxanthine	0	0	0
1-Methyl Xanthine	0	0	0
3-Methyl Xanthine	0	0	0
7-Methyl Xanthine	0	0	0
8-Methyl Xanthine	0	0	0
1.3-Dimethyl Xanthine[b]	0	0	0
1,7-Dimethy Xanthine[c]	0	100	0
3,7-Dimethyl Xanthine[d]	0	0	0
Uric acid	0	0	0
1-Methyluric acid	0	0	0
3-Methyluric acid	0	0	0
7-Methyluric acid	0	0	0
1,3-Dimethyluric acid	0	0	0
1,7-Dimethyluric acid	0	0	100
3,7-Dimethyluric acid	0	0	0
1,3,7-Trimethyluric acid	0	0	0
Guanine	0	0	0
Uracil	0	0	0
AAU[e]	0	0	0
AAMU[f]	0	0	0

| AADMU[g] | 0 | 0 | 0 |

a, The number 0 indicates either an absence of inhibition or an inhibition no higher than 40% at the highest concentration tested in the ELISA (5×10^{-3} M); concentrations of caffeine, 1,7-Dimethyl Xanthine and 1,7-Dimethyluric acid required for 50% inhibition in the competitive antigen ELISA will be determined; b, 1,3-Dimethyl Xanthine, theophylline; c, 1,7-Dimethyl Xanthine, paraxanthine; d, 3,7-Dimethyl Xanthine, theobromine; e, AAU, 5-acetamido-6-aminouracil; f, AAMU, 5-acetamido-6-amino-3-methyluracil; f, AADMU, 5-acetamido-6-amino-1,3-dimethylxanthine.

RESULTS

Positive creation of antibodies against caffeine, 1,7-DMX, and 1,7-DMU can be seen by antibody titers of 30,000-100,000 as determined by the ELISA, strong precipitation lines after double immunodiffusion in agar plates of antisera and derivatives conjugated to rabbit serum albumin, and low cross-reactivity with other caffeine derivatives. These results constitute positive conditions for the development of a competitive antigen ELISA according to the methods described in the above section entitled Materials and Methods.

In accordance with one embodiment of the present invention, a competitive antigen ELISA will be developed for CYP 1A2 phenotyping using caffeine as the probe drug. Contrary to current methods used for phenotyping, the assay is sensitive, rapid and can be readily carried out on a routine basis by a technician with a minimum of training in a clinical laboratory.

Determination of Caffeine, 1,7-Dimethyl Xanthine (1,7-DMX) and 1,7-Dimethyluric acid (1,7-DMU) in urine samples with the ELISA kit

Table 6

Content of the ELISA kit and conditions of storage

Item	Unit	State	Amt.	Storage Conditions
Tween™ 20	1 vial	liquid	250 µL/vial	4°C
H_2O_2	1 vial	liquid	250 µL/vial	4°C
Caffeine-HRP	1 vial	liquid	250 µL/vial	4°C
1,7-DMX-HRP	1 vial	liquid	250 µL/vial	4°C
1,7-DMU-HRP	1 vial	liquid	250 µL/vial	4°C
Buffer A	4 vials	Solid	0.8894 g /vial	4°C
Buffer B	6 vials	Solid	1.234 g/vial	4°C
Buffer C	6 vials	Solid	1.1170 g/vial	4°C
Buffer D	6 vials	Solid	0.8082 g/vial	4°C
Plate (Caffeine-Ab)	2	Solid	-	4°C
Plate (1,7-DMX-Ab)	2	Solid	-	4°C
Plate (1,7-DMU-Ab)	2	Solid	-	4°C
Buffer E	6 vials	Solid	0.9567 g/vial	-20°C
Standards (Caffeine)	14 vials	Liquid	200 µL	-20°C
Standards (1,7-DMX)	14 vials	Liquid	200 µL	-20°C
Standards (1,7-DMU)	14 vials	Liquid	200 µL	-20°C
1N NaOH	1 bottle	Liquid	15 mL	20°C
1N HCl	1 bottle	Liquid	15 mL	20°C

Dilutions of urine samples for the determinations of [Caffeine], [1,7-DMX] and [1X] by ELISA

The dilutions of urine samples required for determinations of caffeine, 1,7-DMX and 1,7-DMU are a function of the sensitivity of the competitive antigen ELISA and of caffeine, 1,7-DMX and 1,7-DMU

concentrations in urine samples. It is suggested to dilute the urine samples by a factor so that AAMU and 1X are about 3×10^{-6} M in the well of the microtiter plate.

Table 7

Dilution Factor	Microtube #							
	20x	40x	50x	80x	100x	150x	200x	400x
Solution	1	2	3	4	5	6	7	8
Urine Sample (mL)[a] 10x diluted	500	250	200	125	100	66.7	50	25
Buffer B (mL)	500	750	800	875	900	933.3	950	975

a: Vortex the microtubes containing the urine sample before pipet-ting.

Store the diluted urine samples at -20°C in a box for microtubes.

Buffer B: dissolve the content of 1 vial B/ 100mL

Determination of [caffeine], [1,7-DMX] and [1,7-DMU] in diluted urine samples by ELISA

Precautions

The substrate is carcinogenic. Wear surgical gloves when handling Buffer E (substrate buffer). Each sample is determined in duplicate. An excellent pipeting technique is required. When this technique is mastered the absorbency values of duplicates should be within less than 5%. Buffers C, D, E are freshly prepared. Buffer $E-H_2O_2$ is prepared just prior to pipeting in the microtiter plate wells.

Preparation of samples:

Prepare table 8 with a computer and print it. This table shows the contents of each well of a 96 well microtiter plate. Enter the name of the urine sample (or number) at the corresponding well positions in

Table 8. Select the dilution factor (D.F.) of each
urine sample and enter at the corresponding position in
Table 8. Enter the dilution of each urine sample with
buffer B at the corresponding position in Table 8: for
5   example a D.F. of 100 (100μL of 10x diluted urine
sample + 900 μL buffer B), enter 100/900. See
"Dilutions of urine samples…" procedure described above
for the preparation of the different dilutions. Prepare
the different dilutions of the urine samples in 1.5 mL
10   microtubes using a styrofoam support for 100
microtubes. Prepare Table 9 with a computer and print
it. Using a styrofoam support (100 microtubes), prepare
the following 48 microtubes in the order indicated in
Table 9.

Table 8

Positions of blanks, control and urine samples in a
microtiter plate

Sample	Well #	D.F.	Dil.	Sample	Well #	D.F.	Dil.
Blank	1-2	-		Control	49-50		-
Control	3-4	-		8	51-52		
S1	5-6	-		9	53-54		
S2	7-8	-		10	55-56		
S3	9-10	-		11	57-58		
S4	11-12	-		12	59-60		
S5	13-14	-		13	61-62		
S6	15-16	-		14	63-64		
S7	17-18	-		15	65-66		
S8	19-20	-		16	67-68		
S9	21-22	-		17	69-70		
S10	23-24	-		Control	71-72		-
S11	25-26	-		18	73-74		
S12	27-28	-		19	75-76		
S13	29-30	-		20	77-78		
S14	31-32	-		21	79-80		
S15	33-34	-		22	81-82		
1	35-36			23	83-84		
2	37-38			24	85-86		
3	39-40			25	87-88		
4	41-42			26	89-90		
5	43-44			27	91-92		
6	45-46			28	93-94		
7	47-48			Blank	95-96		-

Table 9

Content of the different microtubes

Tube #	Sample	Content	Tube #	Sample	Content
1	Blank	Buffer B	25	7	Dil. Urine
2	Control	Buffer B	26	8	Dil. Urine
3	S1	Caffeine/1,7-DMX/1,7-DMU	27	9	Dil. Urine
4	S2	Caffeine/1,7-DMX/1,7-DMU	28	10	Dil. Urine
5	S3	Caffeine/1,7-DMX/1,7-DMU	29	11	Dil. Urine
6	S4	Caffeine/1,7-DMX/1,7-DMU	30	12	Dil. Urine
7	S5	Caffeine/1,7-DMX/1,7-DMU	31	13	Dil. Urine
8	S6	Caffeine/1,7-DMX/1,7-DMU	32	14	Dil. Urine
9	S7	Caffeine/1,7-DMX/1,7-DMU	33	15	Dil. Urine
10	S8	Caffeine/1,7-DMX/1,7-DMU	34	16	Dil. Urine
11	S9	Caffeine/1,7-DMX/1,7-DMU	35	17	Dil. Urine
12	S10	Caffeine/1,7-DMX/1,7-DMU	36	Control	Buffer B
13	S11	Caffeine/1,7-DMX/1,7-DMU	37	18	Dil. Urine
14	S12	Caffeine/1,7-DMX/1,7-DMU	38	19	Dil. Urine
15	S13	Caffeine/1,7-DMX/1,7-DMU	39	20	Dil. Urine
16	S14	Caffeine/1,7-DMX/1,7-DMU	40	21	Dil. Urine
17	S15	Caffeine/1,7-DMX/1,7-DMU	41	22	Dil. Urine
18	1	Dil. Urine	42	23	Dil. Urine
19	2	Dil. Urine	43	24	Dil. Urine
20	3	Dil. Urine	44	25	Dil. Urine
21	4	Dil. Urine	45	26	Dil. Urine
22	5	Dil. Urine	46	27	Dil. Urine
23	6	Dil. Urine	47	28	Dil. Urine
24	Control	Buffer B	48	Blank	Buffer B

Solutions

Buffer C: Dissolve the content of one vial C/50 mL.
                 Pipet 25 mL of Tween™ 20.

Buffer D: Dissolve the content of one vial D/25 mL.
                 Pipet 25 mL of Tween™ 20.

0.05% Tween™ 20:   Pipet 25 mL of Tween™ 20 in a 100
mL erlenmeyer flask containing 50 mL of water.

2.5N HCl: 41.75 mL of 12N HCl/200 mL. Store in a 250 mL
glass bottle

5   Caffeine-HRP conjugate: Pipet 9 mL of Buffer C in a 15
mL glass test tube. Pipet 90 µL of caffeine-HRP stock
solution.

1,7-DMX-HRP conjugate: Pipet 9 mL of Buffer C in a 15
mL glass test tube. Pipet 90 µL of 1,7-DMX-HRP stock

10   solution.

1,7-DMU-HRP conjugate: Pipet 9 mL of the 2% BSA solu-
tion in a 15 mL glass test tube. Pipet 90 µL of 1,7-
DMU-HRP stock solution.

Buffer E – H_2O_2:   Dissolve the contents of 1 vial E-

15   substrate/50 mL water. Pipet 25 µL of a 30% H_2O_2
solution (prepared fresh).

Table 10

Standard solutions of caffeine, 1,7-DMX and 1,7-DMU
(diluted with buffer B)

Standard	Caffeine	Standard	1,7-DMX	Standard	1,7-DMU
1	1.12×10^{-4} M	1	1.12×10^{-4} M	1	1.12×10^{-4} M
2	6.00×10^{-5} M	2	6.00×10^{-5} M	2	6.00×10^{-5} M
3	3.56×10^{-5} M	3	3.56×10^{-5} M	3	3.56×10^{-5} M
4	2.00×10^{-5} M	4	2.00×10^{-5} M	4	2.00×10^{-5} M
5	6.00×10^{-6} M	5	6.00×10^{-6} M	5	6.00×10^{-6} M
6	3.56×10^{-6} M	6	3.56×10^{-6} M	6	3.56×10^{-6} M
7	2.00×10^{-6} M	7	2.00×10^{-6} M	7	2.00×10^{-6} M
8	1.12×10^{-6} M	8	1.12×10^{-6} M	8	1.12×10^{-6} M
9	6.00×10^{-7} M	9	6.00×10^{-7} M	9	6.00×10^{-7} M
10	3.56×10^{-7} M	10	3.56×10^{-7} M	10	3.56×10^{-7} M
11	2.00×10^{-7} M	11	2.00×10^{-7} M	11	2.00×10^{-7} M
12	1.12×10^{-7} M	12	1.12×10^{-7} M	12	1.12×10^{-7} M
13	6.00×10^{-8} M	13	6.00×10^{-9} M	13	6.00×10^{-8} M
14	3.56×10^{-8} M	14	3.56×10^{-8} M	14	3.56×10^{-8} M
15	2.00×10^{-8} M	15	2.00×10^{-8} M	15	2.00×10^{-8} M

Conditions of the ELISA

Pipet 50 µL/well of Caffeine-HRP (1,7-DMX-HRP or 1,7-DMU-HRP) conjugate solution starting from the last row. Pipet 50 µL/well of diluted urine samples in duplicate, standards, blank with a micropipet (0-200 µL), starting from well # 96 (see Table 11). Cover the plate and mix gently by vortexing for several seconds. Leave the plate at room temperature for 3 hours. Wash three times with 100 µL/well buffer C, using a microtiter plate washer. Wash 3 times with 100 µL/well 0.05% Tween™ 20 solution. Pipet 150 µL/well of Buffer E - H_2O_2 (prepared just prior to pipeting in the microtiter plate wells). Shake for 20-30 min. at room

temperature using an orbital shaker. Pipet 50 μL/well
of a 2.5N HCl solution. Shake 3 min. with the orbital
shaker at room temperature. Read the absorbance of the
wells with a microtiter plate reader at 490 nm. Print
5   the sheet of data and properly label.

**Calculation of the [caffeine], [1,7-DMX] and [1,7-DMU]
in urine samples from the data**
        Draw table 11 with a computer. Using the data
    of the microtiter plate reader, enter the average
10 absorbance values of blanks, controls (no free hapten
    present), standards and samples in Table 11. Draw the
    calibration   curve   on   a   semi-logarithmic   plot
    (absorbance at 490 nm as a function of the standard
    concentrations)   using   sigma-plot   (or   other   plot
15 software). Find the [AAMU] (or [1X]) in the microtiter
    well of the unknowns from the calibration curve and
    enter the data in Table 12. Multiply the [caffeine]
    ([1,7-DMX] or [1,7-DMU] of the unknown by the dilution
    factor and enter the result in the corresponding cell
20 of Table 12.

Table 11

Average absorbance values of samples in the microtiter plate

Sample	Well #	A_{490}	Sample	Well #	A_{490}
Blank	1-2		Control	49-50	
Control	3-4		8	51-52	
S1	5-6		9	53-54	
S2	7-8		10	55-56	
S3	9-10		11	57-58	
S4	11-12		12	59-60	
S5	13-14		13	61-62	
S6	15-16		14	63-64	
S7	17-18		15	65-66	
S8	19-20		16	67-68	
S9	21-22		17	69-70	
S10	23-24		Control	71-72	
S11	25-26		18	73-74	
S12	27-28		19	75-76	
S13	29-30		20	77-78	
S14	31-32		21	79-80	
S15	33-34		22	81-82	
1	35-36		23	83-84	
2	37-38		24	85-86	
3	39-40		25	87-88	
4	41-42		26	89-90	
5	43-44		27	91-92	
6	45-46		28	93-94	
7	47-48		Blank	95-96	

Table 12

caffeine, 1,7-DMX and 1,7-DMU concentrations in urine
samples

Sample	D.F.	[Caffeine]	[caffeine] x D.F.
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			
11			
12			
13			
14			
15			
16			
17			
18			
19			
20			
21			
22			
23			
24			
25			
26			
27			
28			
29			

Table 13

Composition of the different buffers

Buffer	pH	Composition	Conc. (mM)	[P] (mM)
A	7.50	0.15629 g/100 mL NaH$_2$PO$_4$ 1.622 g/100 mL Na$_2$HPO$_4$.7H$_2$O 1.778 g/100 mL (total weight)	11.325 60.099	71.424
B	7.50	0.1210191 g/100 mL NaH$_2$PO$_4$ 1.11309 g/100 mL Na$_2$HPO$_4$.7H$_2$O 1.2341 g/100 mL (total weight)	8.769 41.23	49.999
C	7.50	1 g/ 100mL BSA 0.1210191 g/100 mL NaH$_2$PO$_4$ 1.11309 g/100 mL Na$_2$HPO$_4$.7H$_2$O 2.2341 g/100 mL (total weight)	8.769 41.23	49.999
D	7.50	2 g/ 100mL BSA 0.1210191 g/100 mL NaH$_2$PO$_4$ 1.11309 g/100 mL Na$_2$HPO$_4$.7H$_2$O 3.2341 g/100 mL (total weight)	8.769 41.23	49.999
E	5.00	0.52508 g/ 100mL of citric acid 1.34848 g/100 mL Na$_2$HPO$_4$.7H$_2$O 40 mg/100 mL of o-phenylenedi- amine hydrochloride 1.913567 g/100 mL (total weight)	25 50	-

While the invention has been described in
connection with specific embodiments thereof, it will
be understood that it is capable of further
modifications and this application is intended to cover
any variations, uses, or adaptations of the invention
following, in general, the principles of the invention

and including such departures from the present
disclosure as come within known or customary practice
within the art to which the invention pertains and as
may be applied to the essential features hereinbefore
5   set forth, and as follows in the scope of the appended
claims.

WHAT IS CLAIMED IS:

1. A method of determining CYP 1A2 phenotype of an individual which comprises measuring molar ratio of caffeine and first and second different metabolites of caffeine in a biological sample of said individual after drinking a caffeine solution with at least one antibody specific to caffeine and two antibodies each specific to a different metabolite of caffeine, wherein a molar ratio of 4 is indicative of slow intermediate and of 12 is indicative of fast CYP 1A2 metabolizers; and whereby said molar ratio is indicative of a CYP 1A2 phenotype of said individual.

2. The method of claim 1, wherein said first caffeine metabolite is selected from the group consisting of 1,7-dimethylxanthine (1,7 DMX), and those illustrated in Fig. 3; wherein said second caffeine metabolite is selected from the group consisting of 1,7-dimethyluric acid (1,7 DMU), and those illustrated in Fig. 4; and wherein said third metabolite is selected from the group consisting of 1,3,7-trimethylxanthine (caffeine) and those illustrated in Fig. 2.

3. The method of claim 2, wherein said biological sample is urine sample.

4. The method of claim 3, wherein said determined CYP 1A2 phenotype of said individual allows physician to predict susceptibility to carcinogen induced disease and/or to individualize drug treatments.

5.   A competitive enzyme linked immunosorbent assay
(ELISA) method for determining CYP 1A2 phenotype, which
comprises using at least one antibody specific to
caffeine and two antibodies each specific to a
different metabolite of caffeine to measure their molar
ratio in biological sample of an individual after
drinking a caffeine solution; wherein a molar ratio of
4 is indicative of slow intermediate and of 12 is
indicative of fast CYP 1A2 metabolizers; and whereby
said molar ratio is indicative of a CYP 1A2 phenotype
of said individual.

6.   The ELISA method of claim 5, wherein said first
caffeine metabolite is selected from the group
consisting of 1,7-dimethylxanthine (1,7 DMX), and those
illustrated in Fig. 3; wherein said second caffeine
metabolite is selected from the group consisting of
1,7-dimethyluric acid (1,7 DMU), and those illustrated
in Fig. 4; and wherein said third metabolite is
selected   from   the   group   consisting   of
1,3,7-trimethylxanthine   (caffeine),   and   those
illustrated in Fig. 2.

7.   The ELISA method of claim 6, wherein said
biological sample is urine sample.

8.   The ELISA method of claim 7, wherein the
determined CYP 1A2 phenotype of said individual allows
a physician to predict susceptibility to carcinogen
induced diseases and/or to individualize drug
treatments.

9.   A competitive enzyme linked immunosorbent assay
(ELISA) kit for determining CYP 1A2 phenotype, which

comprises at least one antibody specific to caffeine and two antibodies each specific to a different metabolite of caffeine to measure their molar ratio in biological sample of an individual after drinking a caffeine solution; wherein a molar ratio of 4 is indicative of slow intermediate and of 12 is indicative of fast CYP 1A2 metabolizers; and whereby said molar ratio is indicative of a CYP 1A2 phenotype of said individual.

10.   The competitive ELISA kit of claim 9, further comprises:

a)   a plate coated with a first antibody specific to caffeine;

b)   a second antibody specific to a first metabolite of caffeine;

c)   a third antibody specific to a second metabolite of caffeine;

d)   a known amount of caffeine-horseradish peroxidase conjugate wherein a standard calibration curve is obtained;

e)   a known amount of 1,7-dimethyl xanthine-horseradish peroxidase conjugate wherein a standard calibration curve is obtained; and

f)   a known amount of 1,7-dimethyluric acid-horseradish peroxidase conjugate wherein a standard calibration curve is obtained.

11.   The method of claim 1, wherein said specific antibodies are polyclonal or monoclonal antibodies.

12.   The method of claim 1 wherein said specific antibodies are polyclonal antibodies.

13.   The competitive antigen enzyme linked immunosorbent assay (ELISA) of claim 5 wherein said specific antibodies are polyclonal or monoclonal antibodies.

14.   The competitive antigen enzyme linked immunosorbent assay (ELISA) of claim 5 wherein said specific antibodies are polyclonal antibodies.

15.   The competitive ELISA kit of claim 10 wherein said specific antibodies are polyclonal or monoclonal antibodies.

16.   The competitive ELISA kit of claim 10 wherein said specific antibodies are polyclonal antibodies.

17.   A method of determining NAT1 phenotype of an individual which comprises measuring molar ratio of p-aminosalicylic acid in a biological sample of an individual after consuming p-aminosalicylic acid with at least 2 antibodies each specific to p-aminosalicylic acid or a different metabolite of p-aminosalicylic acid, and whereby said molar ratio is indicative of a NAT1 phenotype of said individual.

18.   The method of claim 17, wherein a first p-aminosalicylic acid metabolite is selected from the group consisting of 4-oxomethyl-aminosalicylic acid and those illustrated in Fig. 1; wherein p-aminosalicylic acid is selected and illustrated in Fig. 1.

19.   The method of claim 18, wherein said biological sample is urine sample.

20.   The method of claim 19, wherein said determined NAT1 phenotype of said individual allows physician to predict susceptibility to carcinogen induced disease and/or individualize drug treatments.

21.   A competitive enzyme linked immunosorbent assay (ELISA) method for determining NAT1 phenotype, which comprises using at least 2 antibodies each specific to p-aminosalicylic acid or a metabolite of p-aminosalicylic acid to measure their molar ratio in biological sample of an individual after consuming p-aminosalicylic acid; and whereby said molar ratio is indicative of a NAT1 phenotype of said individual.

22.   The ELISA method of claim 21, wherein a first p-aminosalicylic acid metabolite is selected from the group consisting of 4-oxomethyl-aminosalicylic acid and those illustrated in Fig. 1; wherein p-aminosalicylic acid is selected and illustrated in Fig. 1.

23.   The ELISA method of claim 22, wherein said bio-logical sample is urine sample.

24.   The ELISA method of claim 23, wherein the deter-mined NAT1 phenotype of said individual allows a physi-cian to predict susceptibility to carcinogen induced diseases and/or to individualize drug treatments.

25.   A competitive enzyme linked immunosorbent assay
(ELISA) kit for determining NAT1 phenotype, which com-
prises at least 2 antibodies each specific to p-amino-
salicylic acid or a metabolite of p-aminosalicylic acid
to measure their molar ratio in a biological sample of
an individual after consuming p-aminosalicylic acid,
and whereby said molar ratio is indicative of a NAT1
phenotype of said individual.

26.   The competitive ELISA kit of claim 25, further
comprises:

a)   a plate coated with a first antibody specific to
       p-aminosalicylic acid;

b)   a second antibody specific to a first metabolite
       of p-aminosalicylic acid;

c)   a   known   amount   of   p-aminosalicylic   acid-
       horseradish   peroxidase   conjugate   wherein   a
       standard calibration curve is obtained; and

d)   a known amount of p-aminosalicylic metabolite-
       horseradish peroxidase conjugate wherein a stan-
       dard calibration curve is obtained.

27.   The method of claim 17 wherein said specific
antibodies are polyclonal or monoclonal antibodies.

28.   The method of claim 17 wherein said specific
antibodies are polyclonal antibodies.

29.   The competitive antigen enzyme linked immunosor-
bent assay (ELISA) of claim 21 wherein said specific
antibodies are polyclonal or monoclonal antibodies.

30.   The competitive antigen enzyme linked immunosor-
bent assay (ELISA) of claim 21 wherein said specific
antibodies are polyclonal antibodies.

31.   The competitive ELISA kit of claim 26 wherein
said specific antibodies are polyclonal or monoclonal
antibodies.

32.   The competitive ELISA kit of claim 26 wherein
said specific antibodies are polyclonal antibodies.

33.   A method of determining CYP 2D6 phenotype of an
individual which comprises measuring molar ratio of
first   and   second   different   metabolites   of
dextromethorphan in a biological sample of said
individual after consuming dextromethorphan with at
least 2 antibodies each specific to dextromethorphan or
a metabolite, wherein a molar ratio >1 is indicative of
slow intermediate and <1 is indicative of fast CYP 2D6
metabolizers;   and   whereby   said   molar   ratio   is
indicative of a CYP 2D6 phenotype of said individual.

34.   The method of claim 33, wherein a first dextro-
methorphan   metabolite   is   selected   from   the   group
consisting of 3 hydroxy-17-methylmorphinan, and those
illustrated in Fig. 5; and dextromethorphan is selected
and illustrated in Fig. 5.

35.   The method of claim 34, wherein said biological
sample is urine sample.

36.   The method of claim 35, wherein the determined CYP 2D6 phenotype of said individual allows physician to predict susceptibility to carcinogen induced disease and/or to individualize drug treatments.

37.   A competitive enzyme linked immunosorbent assay (ELISA) method for determining CYP 2D6 phenotype, which comprises using at least 2 antibodies each specific to dextromethorphan or a metabolite of dextromethorphan to measure their molar ratio in a biological sample of an individual after consuming dextromethorphan, wherein a molar ratio >1 is indicative of slow and a molar ratio <1 is indicative of fast CYP 2D6 metabolizers; whereby said molar ratio is indicative of a CYP 2D6 phenotype of said individual.

38.   The ELISA method of claim 37, is selected from the group consisting of 3-hydroxy-17-methylmorphinan, and those illustrated in Fig. 5, a dextromethorphan is selected and illustrated in Fig. 5.

39.   The ELISA method of claim 38, wherein said biological sample is urine sample.

40.   The   ELISA   method   claim   38,   wherein   the determined CYP 2D6 phenotype of said individual allows a physician to predict susceptibility to carcinogen induced   diseases   and/or   to   individualize   drug treatments.

41.   A competitive enzyme linked immunosorbent assay (ELISA) kit for determining CYP 2D6 phenotype, which

comprises at least 2 antibodies each specific to a
different metabolite of dextromethorphan to measure
their molar ratio in a biological sample of an
individual after consuming dextromethorphan, wherein a
molar ratio >1 is indicative of slow and a molar ratio
<1 is indicative of fast CYP 2D6 metabolizers; whereby
said molar ratio is indicative of a CYP 2D6 phenotype
of said individual.

42.   The competitive ELISA kit of claim 41, further
comprises:

a)   a plate coated with a first antibody specific to
         dextromethorphan;

b)   a second antibody specific to a first metabolite
         of dextromethorphan;

c)   a known amount of dextromethorphan-horseradish
         peroxidase conjugate wherein a standard calibra-
         tion curve is obtained; and

d)   a known amount of dextromethorphan metabolite-
         horseradish peroxidase conjugate wherein a stan-
         dard calibration curve is obtained; and

43.   The method of claim 33 wherein said specific
antibodies are polyclonal or monoclonal antibodies.

44.   The method of claim 33 wherein said specific
antibodies are polyclonal antibodies.

45.   The competitive enzyme linked immunosorbent
assay (ELISA) of claim 37 wherein said specific
antibodies are polyclonal or monoclonal antibodies.

46.   The competitive enzyme linked   immunosorbent
assay (ELISA) of claim 37 wherein said specific
antibodies are polyclonal antibodies.

47.   The competitive ELISA kit of claim 42 wherein
said specific antibodies are polyclonal or monoclonal
antibodies.

48.   The competitive ELISA kit of claim 42 wherein
said specific antibodies are polyclonal antibodies.

49.   A method of determining CYP 2E1 phenotype of an
individual which comprises measuring molar ratio of
first and second different metabolites of chlorzoxazone
in a biological sample of an individual after consuming
chlorzoxazone with at least 2 antibodies, each specific
to a different metabolite of chlorzoxazone, whereby
said molar ratio is indicative of a CYP 2E1 phenotype
of said individual.

50.   The method of claim 49, wherein a first
chlorzoxazone metabolite is selected from the group
consisting of 5-chloro-6-hydroxy-benzoxazole, and those
illustrated in Fig. 6; chlorzoxazone is selected and
illustrated in Fig. 6.

51.   The method of claim 50, wherein said biological
sample is urine sample.

52.   The method of claim 51, wherein the determined
CYP 2E1 phenotype of said individual allows physician

to predict susceptibility to carcinogen induced disease and/or to individualize drug treatments.

53.   A competitive enzyme linked immunosorbent assay (ELISA) method for determining CYP 2E1 phenotype, which comprises using at least 2 antibodies each specific to a different metabolite of chlorzoxazone to measure their molar ratio in a biological sample of an individual after consuming chlorzoxazone; whereby said molar ratio is indicative of a CYP 2E1 phenotype of said individual.

54.   The ELISA method of claim 53, wherein a first chlorzoxazone metabolite is selected from the group consisting of 5-chloro-6-hydroxy-benzoxazole, and those illustrated in Fig. 6, and chlorzoxazone is selected and illustrated in Fig. 6.

55.   The ELISA method of claim 53, wherein said bio-logical sample is urine sample.

56.   The ELISA method claim 53, wherein the determined CYP 2E1 phenotype of said individual allows a physician to predict susceptibility to carcinogen induced diseases and/or to individualize drug treatments.

57.   A competitive enzyme linked immunosorbent assay (ELISA) kit for determining CYP 2E1 phenotype, which comprises at least 2 antibodies each specific to a different metabolite of chlorzoxazone to measure their molar ratio in a biological sample of an individual

after consuming chlorzoxazone; whereby said molar ratio
is indicative of a CYP 2E1 phenotype of said
individual.

58.   The competitive ELISA kit of claim 57, further
comprises:

a)   a plate coated with a first antibody specific to
       chloroxazone;

b)   a second antibody specific to a first metabolite
       of chlorzoxazone;

c)   a known amount of chlorzoxazone -horseradish
       peroxidase conjugate wherein a standard
       calibration curve is obtained; and

d)   a known amount of chlorzoxazone metabolite-
       horseradish peroxidase conjugate wherein a
       standard calibration curve is obtained.

59.   The method of claim 49 wherein said specific
antibodies are polyclonal or monoclonal antibodies.

60.   The method of claim 49 wherein said specific
antibodies are polyclonal antibodies.

61.   The competitive antigen enzyme linked immunosor-
bent assay (ELISA) of claim 53 wherein said specific
antibodies are polyclonal or monoclonal antibodies.

62.   The competitive antigen enzyme linked immunosor-
bent assay (ELISA) of claim 53 wherein said specific
antibodies are polyclonal antibodies.

63.   The competitive ELISA kit of claim 58 wherein
said specific antibodies are polyclonal or monoclonal
antibodies.

64.   The competitive ELISA kit of claim 58 wherein
said specific antibodies are polyclonal antibodies.

65.   A method of determining CYP 3A4 phenotype of an
individual which comprises measuring molar ratio of
first   and   second   different   metabolites   of
dextromethorphan   in   a   biological   sample   of   an
individual after consuming dextromethorphan with at
least 2 antibodies, each specific to dextromethorphan
or a metabolite of dextromethorphan, whereby said molar
ratio is indicative of a CYP 3A4 phenotype of said
individual.

66.   The method of claim 65, wherein a first dextro-
methorphan   metabolite   is   selected   from   the   group
consisting   of   3-methoxy-morphinan,   and   those
illustrated in Fig. 7; and dextromethorphan is selected
and illustrated in Fig. 7.

67.   The method of claim 66, wherein said biological
sample is urine sample.

68.   The method of claim 67, wherein the determined
CYP 3A4 phenotype of said individual allows physician
to predict susceptibility to carcinogen induced disease
and/or to individualize drug treatments.

69.   A competitive enzyme linked immunosorbent assay
(ELISA) method for determining CYP 3A4 phenotype, which
comprises using at least 2 antibodies each specific to
a different metabolite of dextromethorphan to measure
their molar ratio in a biological sample of an
individual after consuming dextromethorphan, whereby
said molar ratio is indicative of a CYP 3A4 phenotype
of said individual.

70.   The ELISA method of claim 69, wherein a first
dextromethorphan metabolite is selected from the group
consisting of 3-methoxymorphinan, and those illustrated
in Fig. 7; and dextromethorphan is selected and
illustrated in Fig. 7.

71.   The ELISA method of claim 69, wherein said bio-
logical sample is urine sample.

72.   The ELISA method claim 69, wherein the
determined CYP 3A4 phenotype of said individual allows
a physician to predict susceptibility to carcinogen
induced diseases and/or to individualize drug
treatments.

73.   A competitive enzyme linked immunosorbent assay
(ELISA) kit for determining CYP 3A4 phenotype, which
comprises at least 2 antibodies each specific to a
different metabolite of dextromethorphan to measure
their molar ratio in a biological sample of an
individual after consuming dextromethorphan, whereby
said molar ratio is indicative of a CYP 3A4 phenotype
of said individual.

74.   The competitive ELISA kit of claim 73, further comprises:

a)   a plate coated with a first antibody specific to dextromethorphan;

b)   a second antibody specific to a first metabolite of dextromethorphan;

c)   a known amount of dextromethorphan-horseradish peroxidase conjugate wherein a standard calibration curve is obtained; and

d)   a known amount of dextromethorphan metabolite-horseradish peroxidase conjugate wherein a standard calibration curve is obtained.

75.   The method of claim 65 wherein said specific antibodies are polyclonal or monoclonal antibodies.

76.   The method of claim 65 wherein said specific antibodies are polyclonal antibodies.

77.   The competitive antigen enzyme linked immunosorbent assay (ELISA) of claim 69 wherein said specific antibodies are polyclonal or monoclonal antibodies.

78.   The competitive antigen enzyme linked immunosorbent assay (ELISA) of claim 69 wherein said specific antibodies are polyclonal antibodies.

79.   The competitive ELISA kit of claim 74 wherein said specific antibodies are polyclonal or monoclonal antibodies.

80.   The competitive ELISA kit of claim 74 wherein said specific antibodies are polyclonal antibodies.

81.   A   p-aminosalicylic   acid   derivative   as illustrated in Figure 1.

82.   A p-aminosalicylic acid metabolite derivative as illustrated in Figure 1.

83.   A   caffeine   derivative   as   illustrated   in Figure 2.

84.   A 1,7-dimethylxanthine metabolite derivative as illustrated in Figure 3.

85.   A 1,7-dimethyluric acid metabolite derivative as illustrated in Figure 4.

86.   A dextromethorphan derivative as illustrated in Figure 5.

87.   A   dextromethorphan   metabolite   derivative   as illustrated in Figure 5.

88.   A chlorzoxazone   derivative   as illustrated   in Figure 6.

89.   A   chlorzoxazone   metabolite   derivative   as illustrated in Figure 6.

90.   A dextromethorphan derivative as illustrated in Figure 7.

91.   A   dextromethorphan   metabolite   derivative   as illustrated in Figure 7.

92.   A method of synthesizing a caffeine derivative comprising synthesizing said caffeine derivative according to a caffeine synthetic pathway as illustrated in Figure 8.

93.   A method of synthesizing a 1,7-dimethylxanthine derivative comprising synthesizing said 1,7-dimethylxanthine derivative according to a 1,7-dimethylxanthine synthetic pathway as illustrated in Figure 8.

94.   A method of synthesizing a caffeine derivative comprising synthesizing said caffeine derivative according to a caffeine synthetic pathway as illustrated in Figure 9.

95.   A method of synthesizing a 1,7-dimethyluric acid derivative comprising synthesizing said 1,7-dimethyluric acid derivative according to a 1,7-dimethyluric acid synthetic pathway as illustrated in Figure 9.

$HOOC$—⟨benzene ring⟩—NH_2
HO

p-aminosalicylic acid

$HOOC$—⟨benzene ring⟩—$NH-(CH_2)n$——X
HO
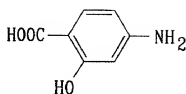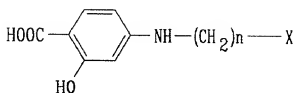
p-aminosalicylic acid derivative I

where $X = NH_2$

$(CH_2)_n NH_2$

$N=C=S$

I

where $n = 1-5$

$HOOC$—⟨benzene ring⟩—$NH-\overset{\overset{O}{\|}}{C}-CH_2-Y$
HO
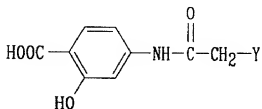
p-aminosalicylic acid derivative II

where $Y = (CH_2)_n NH_2$

$(CH_2)_n N=C=S$

$(CH_2)_n OH$

$(CH_2)_n I$

where $n = 1-5$

FIG - 1

where X = $(CH_2)_n COOH$ n=1-5

or

$(CH_2)_n NH_2$ n=1-5

or

$(CH_2)_n OH$ n=2-5

or

$(CH_2)_n NHCOCH_2CH_2COOH$ n=1-5

or

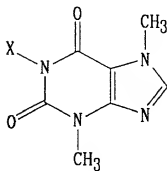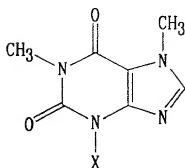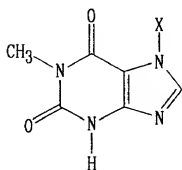$(CH_2)_n OCOCH_2CH_2COOH$ n=2-5

or

$(CH_2)_n N=C=S$ n=1-5

FIG. 2

where X = $(CH_2)_n COOH$ n=1-5

or

$(CH_2)_n NH_2$ n=1-5

or

$(CH_2)_n OH$ n=2-5

or

$(CH_2)_n NHCOCH_2CH_2COOH$ n=1-5

or

$(CH_2)_n OCOCH_2CH_2COOH$ n=2-5

or

$(CH_2)_n N=C=S$ n=1-5

Fig. 3

where X = $(CH_2)_nCOOH$ $n=1-5$

or

$(CH_2)_nNH_2$ $n=1-5$

or

$(CH_2)_nOH$ $n=2-5$

or

$(CH_2)_nNHCOCH_2CH_2COOH$ $n=1-5$

or
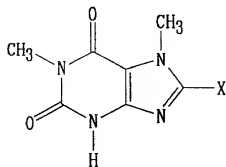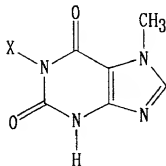
$(CH_2)_nOCOCH_2CH_2COOH$ $n=2-5$

or

$(CH_2)_nN=C=S$ $n=1-5$

FIG. 4

Dextromethorphan

Dextromethorphan derivative I

Dextromethorphan derivative II

where $X = (CH_2)_n COOH$

$(CH_2)_n NH_2$

$(CH_2)_n NHCOCH_2 CH_2 COOH$

$(CH_2)_n N=C=S$

$(CH_2)_n OH$

$(CH_2)_n OCOCH_2 CH_2 COOH$

$CH_2 I$

where $n = 1-5$

FIG. 5

Chlorzoxazone

Chlorzoxazone derivative I

Chlorzoxazone derivative II

where $X = (CH_2)_nNH_2$

$(CH_2)_nN=C=S$

$(CH_2)_nOH$

$(CH_2)_nOCO-CH_2CH_2COOH$

$(CH_2)_nI$

where $n = 0-5$

$\boxed{FIG.6}$

Dextromethorphan

where X = $(CH_2)_n COOH$

$(CH_2)_n NH_2$

$(CH_2)_n NHCOCH_2 CH_2 COOH$

$(CH_2)_n N=C=S$

$(CH_2)_n OH$

$(CH_2)_n OCOCH_2 CH_2 COOH$

$CH_2 I$

where n = 1-5

Dextromethorphan derivative I

Dextromethorphan derivative II

FIG. 7
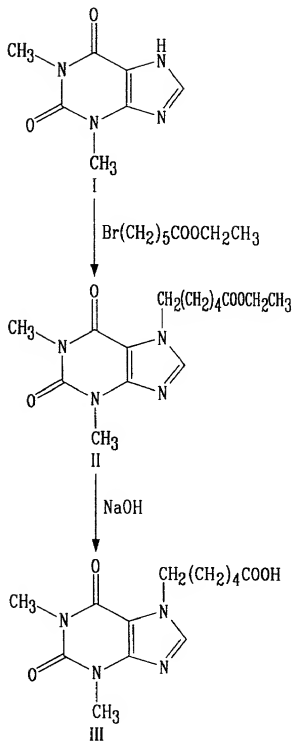
Caffeine derivative

1,7-dimethylxanthine derivative

Fig. 8
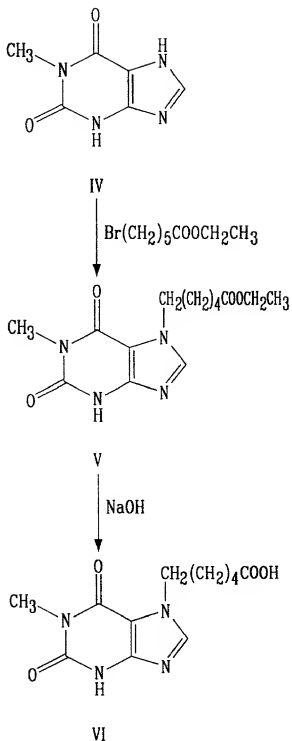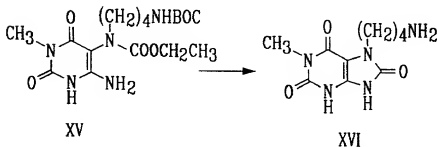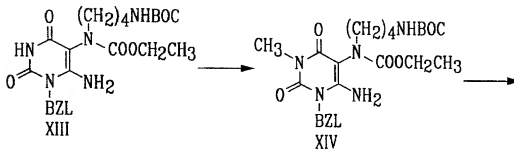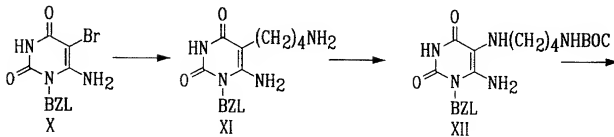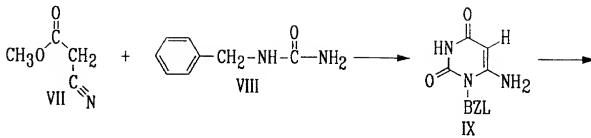
1,7-dimethyluric acid derivative

FIG. 9

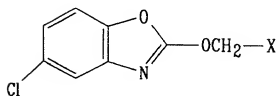|	1	2	3	4	5	6	7	8	9	10	11	12
A	Blk	STD8	STD16	S1	S9	S5	S1	S9	S5	Blk	STD8	STD16
B	STD1	STD9	STD17	S2	S10	S6	S2	S10	S6	STD1	STD9	STD17
C	STD2	STD10	STD18	S3	S11	S7	S3	S11	S7	STD2	STD10	STD18
D	STD3	STD11	STD19	S4	S12	S8	S4	S12	S8	STD3	STD11	STD19
E	STD4	STD12	STD20	S5	S1	S9	S5	S1	S9	STD4	STD12	STD20
F	STD5	STD13	STD21	S6	S2	S10	S6	S2	S10	STD5	STD13	STD21
G	STD6	STD14	STD22	S7	S3	S11	S7	S3	S11	STD6	STD14	STD22
H	STD7	STD15	STD23	S8	S4	S12	S8	S4	S12	STD7	STD15	STD23

FIG. 10

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Declaration for Patent Application

As a named inventor, I hereby declare that:

My residence, mailing address and citizenship are as stated next to my name;

I believe I am the original, first and sole inventor (if only one name is listed) or an original, first and joint inventor (if plural names are listed in the signatory page(s) commencing at page 3 hereof) of the subject matter which is claimed and for which a patent is sought on the invention entitled

ELISA KIT FOR THE DETERMINATION OF METABOLIC PHENOTYPES

the specification of which (check one)

[ ]   is attached hereto.

[X]   is United States Application Number 09/936,507, which was deposited on September 12, 2001, and is the United States National Stage application of International Application Number PCT/CA00/00246, which was filed on March 9, 2000.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 C.F.R. §1.56, including for continuation-in-part applications, material information which became available between the filing date of the prior application and the national or PCT international filing date of the continuation-in-part application.

I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or of any PCT international application having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s)			Priority Not Claimed	Certified Copy Filed? YES NO
_____ (Number)	_____ (Country)	_____ (Day/Month/Year filed)	[ ]	[ ] [ ]
_____ (Number)	_____ (Country)	_____ (Day/Month/Year filed)	[ ]	[ ] [ ]
_____ (Number)	_____ (Country)	_____ (Day/Month/Year filed)	[ ]	[ ] [ ]

I hereby claim the benefit under 35 U.S.C. §119(e) of any United States provisional application(s) listed below.

60/124,488 (Application Number)	March 15,1999 (Filing Date)
_____ (Application Number)	_____ (Filing Date)

I hereby claim the benefit under 35 U.S.C. 120 of any United States application(s), or 365(c) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. 112, I acknowledge the duty to disclose information known by me to be material to patentability as defined in 37 C.F.R. 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

(Application Serial No.)	(Filing date)	(Status: patented, pending, abandoned)
(Application Serial No.)	(Filing date)	(Status: patented, pending, abandoned)
(Application Serial No.)	(Filing date)	(Status: patented, pending, abandoned)
(Application Serial No.)	(Filing date)	(Status: patented, pending, abandoned)

As a named inventor, I hereby appoint the attorneys and/or agents associated with **Hamilton, Brook, Smith & Reynolds, P.C., Two Militia Drive, Lexington, Massachusetts 02421-4799 Customer No. 21005,**

and _____

to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith.
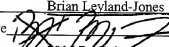
Please send correspondence to:

[X] **Customer No.**   21005
                                HAMILTON, BROOK, SMITH & REYNOLDS, P.C.
                                Two Militia Drive
                                Lexington, MA 02421-4799

or

[X] **Address as follows:**

Direct telephone calls to: David E. Brook, Esq.   Telephone No.: 781-861-6240

Direct facsimiles to: David E. Brook, Esq.   Facsimile No.: 781-861-9540

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of sole
  or first inventor   Brian Leyland-Jones
Inventor's Signature   _____ Date Oct 25, 2001
Residence   7816 Bodinier   Suite 2000 80 S. W. 8th Street
                   Anjou, Quebec H1K 4C5 CAX Meami, FLA   33130
Citizenship   Canadian   USA
Mailing Address   Same as above

Full name of second joint

  inventor, if any _____ Pierre Wong

Inventor's Signature _____ _Pierre Wong_____ Date _October 3, 2001_

Residence _____ 3415 Aylmer, Apartment 4

                   Montreal, Quebec H2X 2B4 CAX

Citizenship _____ Canadian

Mailing Address _____ Same as above

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Declaration for Patent Application

As a named inventor, I hereby declare that:

My residence, mailing address and citizenship are as stated next to my name;

I believe I am the original, first and sole inventor (if only one name is listed) or an original, first and joint inventor (if plural names are listed in the signatory page(s) commencing at page 3 hereof) of the subject matter which is claimed and for which a patent is sought on the invention entitled

____ELISA KIT FOR THE DETERMINATION OF METABOLIC PHENOTYPES_____

the specification of which (check one)

[ ]   is attached hereto.

[X]   is United States Application Number 09/936.507, which was deposited on September 12, 2001, and is the
        United States National Stage application of International Application Number PCT/CA00/00246, which
        was filed on March 9, 2000.

        I hereby state that I have reviewed and understand the contents of the above-identified specification,
including the claims, as amended by any amendment referred to above.

        I acknowledge the duty to disclose information which is material to patentability as defined in 37 C.F.R.
§1.56, including for continuation-in-part applications, material information which became available between the
filing date of the prior application and the national or PCT international filing date of the continuation-in-part
application.

        I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or 365(b) of any foreign application(s)
for patent or inventor's certificate, or 365(a) of any PCT international application which designated at least one
country other than the United States of America, listed below and have also identified below, by checking the box,
any foreign application for patent or inventor's certificate, or of any PCT international application having a filing
date before that of the application on which priority is claimed:

	Prior Foreign Application(s)		Priority Not Claimed	Certified Copy Filed? YES NO
			[ ]	[ ] [ ]
(Number)	(Country)	(Day/Month/Year filed)		
			[ ]	[ ] [ ]
(Number)	(Country)	(Day/Month/Year filed)		
			[ ]	[ ] [ ]
(Number)	(Country)	(Day/Month/Year filed)		

I hereby claim the benefit under 35 U.S.C. §119(e) of any United States provisional application(s) listed below.

____60/124,488_____   ____March 15,1999_____
(Application Number)   (Filing Date)

_____   _____
(Application Number)   (Filing Date)

I hereby claim the benefit under 35 U.S.C. 120 of any United States application(s), or 365(c) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. 112, I acknowledge the duty to disclose information known by me to be material to patentability as defined in 37 C.F.R. 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

(Application Serial No.)	(Filing date)	(Status: patented, pending, abandoned)
(Application Serial No.)	(Filing date)	(Status: patented, pending, abandoned)
(Application Serial No.)	(Filing date)	(Status: patented, pending, abandoned)
(Application Serial No.)	(Filing date)	(Status: patented, pending, abandoned)

As a named inventor, I hereby appoint the attorneys and/or agents associated with
Hamilton, Brook, Smith & Reynolds, P.C., Two Militia Drive, Lexington, Massachusetts 02421-4799
Customer No. 21005,

and _____,

to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith.

Please send correspondence to:

[X] Customer No.   21005
                         HAMILTON, BROOK, SMITH & REYNOLDS, P.C.
                         Two Militia Drive
                         Lexington, MA 02421-4799

or

[X] **Address as follows:**

Direct telephone calls to: _David E. Brook, Esq._____ Telephone No.: 781-861-6240_____

Direct facsimiles to: _____David E. Brook, Esq._____ Facsimile No.: 781-861-9540_____

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of sole
  or first inventor _____Brian Leyland-Jones_____

Inventor's Signature_____ Date _____

Residence _____7816 Bodinier_____

_____Anjou, Quebec H1K 4C5_____

Citizenship _____ ' ___Canadian_____

Mailing Address _____._____Same as above_____

Full name of second joint

  inventor, if any _____ Pierre Wong _____

Inventor's Signature_____ Date _____

Residence _____ 3415 Aylmer, Apartment 4 _____

                       Montreal, Quebec H2X 2B4 _____

Citizenship _____ Canadian _____

Mailing Address _____ Same as above _____